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Na^+ and K^+ Transport in Rat Thymic and Human Leukemic and Non-Leukemic Lymphocytes

Anthony H. Jackson

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Na^+ and K^+ Transport in Rat Thymic and Human Leukemic and Non-Leukemic Lymphocytes

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(A.B., Univ. of Rochester, 1968)

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of

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TO

Mother

and

Dad

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TABLE OF CONTENTS

Introduction 1

Methods 11

Materials 22

Calculations 23

Results 31

Discussion 42

Summary 52

References 54

INTRODUCTION

The study of the movements of monovalent cations across cell membranes has been neglected in leukemia research. Membrane transport in the lymphocyte has been only touched upon despite its potential importance as a component of cell proliferation and immuno-reactive processes. These studies were carried out in order to characterize Na^+ and K^+ membrane transport in lymphocytes.

Over the past two decades, the movements of the monovalent cations, notably sodium and potassium ions, across cell membranes has been studied extensively in many tissues and human red cells in particular. In 1960, Post, Merritt, Kinsolving, and Albright (1) presented evidence for the participation of membrane adenosinetriphosphatase (ATPase), in the transport of sodium and potassium ions. This enzyme, by catalyzing the hydrolysis of adenosine triphosphate (ATP), tranduces the energy for the active influx of potassium and active extrusion of sodium ions in the red cell. Four years later, Sen and Post (2) proposed that, for every high-energy phosphate bond of ATP cleaved by this ATPase, three sodium ions were extruded and two potassium ions accumulated. By the late 1960's, most investigators in the field believed that the

plasma membrane of most, if not all, mammalian cells possessed a (Na^+-K^+)-activated ATPase that catalyzed the transmembranous movement of sodium and potassium ions against their respective concentration gradients. Inhibition of active sodium- and potassium-ion transport by the cardiac glycoside ouabain (g-Strophanthin) was discovered to be such a universal occurrence in cells in which the ATPase had been shown to be present in other ways that this finding alone was generally agreed to define the presence of this enzyme in the cell membrane (3 - 9). Oligomycin, like ouabain, was found to inhibit active Na^+ and K^+ membrane transport in erythrocytes (10 - 12). According to Whittam, Wheeler, and Blake (10), both ouabain and oligomycin act similarly in inhibiting the K^+-activated hydrolysis of a phosphorylated intermediate derived from ATP. Thus, to find that, besides ouabain, oligomycin also inhibited sodium- and potassium-ion fluxes across a particular cell membrane would provide strong supporting evidence for the existence of a (Na^+-K^+)-activated ATPase within that cell membrane.

Studies of cation metabolism in leukocytes were few until the late 1940's. In 1949, working with rabbit polymorphonuclear leukocytes, Wilson and Manery (13) demonstrated that the intracellular concentration of sodium and potassium could be influenced

by the concentration of these cations in the medium and that a reciprocity existed between intracellular sodium and potassium contents; that is, the movements of these ions were somehow coupled so that the total monovalent cation concentration within the cell remained relatively constant. In 1954, studies by Hempling (14) concurred with Wilson and Manery on the existence of a reciprocal exchange of sodium and potassium ions across the cell membrane of rabbit granulocytes. Hempling determined that, in cooling a suspension of neutrophils in vitro to 2° C., sodium uptake was exactly reciprocal to potassium depletion and that, in rewarming the suspension to 37° C., there was a reciprocal Na^+-K^+ exchange in the reverse direction. In contradistinction to both Wilson and Manery and Hempling, Elsbach and Schwartz (15), in 1959, reported that the transmembranous fluxes of these two monovalent cations were not linked reciprocally since rabbit granulocytes incubated at 37° C. subsequent to cooling to 4° C. actively reaccumulated potassium but did not demonstrate a net extrusion of sodium. They argued for the presence of a non-reciprocal, uncoupled transport mechanism for sodium and potassium or for the existence of two independent mechanisms. Tenney and Rafter (16), in 1968, isolated an ATPase from intact rabbit leukocytes that was K^+-dependent and ouabain-inhibitable but

without demonstrable Na^+ dependence. Woodin and Wieneke (17, 18), citing the work of Elsbach and Schwartz (see above) as well as their own data demonstrating the existence of a K^+-activated leukocyte membrane phosphatase, suggested that leukocytes possess a potassium pump to regulate intracellular electrolyte concentration but not a sodium pump. However, Ward and Becker (1970) (19), although tending to follow the thinking of Woodin and Wieneke on this matter, stated, after finding that ouabain inhibited neutrophil chemotaxis, that the last word had not been said on the question of whether a Na^+-K^+ coupled pump is present in the leukocyte cell membrane.

Block and Bonting (20) in 1964 and Lichtman and Weed (21) in 1969 approached the problem of the existence and the level of (Na^+-K^+)-activated ATPase activity in leukocytes in a different manner, namely, by assessing the quantity of inorganic phosphate released following incubation of a leukocyte homogenate containing membrane fragments in a physiologic substrate medium containing ATP. All types of human leukocytes were found to have (Na^+-K^+) -activated ATPase activity. Both groups of investigators showed that the (Na^+-K^+)-activated ATPase activity paralleled the degree of morphologic immaturity of the cell type. Leukemic leukocytes such as myeloblasts (immature granulocytes) demonstrated higher

activity than normal (mature) granulocytes. Normal (mature) peripheral blood lymphocytes and the "mature-looking" small lymphocytes from the blood of patients with chronic lymphatic leukemic exhibited no significant difference in their activities when expressed either per kilogram leukocyte dry weight (20) or per milligram leukocyte protein (21). Studies of intracellular monovalent cation contents of human normal and leukemic leukocytes were performed by Rigas (22), Block and Bonting (20), and Lichtman and Weed (21). In contrast to the first two groups of investigators, Lichtman and Weed (21) pointed out the need to evaluate the ratio of the amount of intracellular water per cell to the dry weight of that cell so that the Na and K contents could be expressed in terms of concentration, thus enabling valid comparison between different leukocytes. According to Lichtman and Weed (21), the intracellular potassium ion concentrations in various leukocyte types did not significantly differ whereas the intracellular sodium ion concentration of leukemic myeloblasts averaged some 10 milliequivalents (mEq)/kilogram cell water higher than that of normal polymorphonuclear granulocytes (a mean of 40 as against) 30). Normal and chronic lymphatic leukemic lymphocytes had similar intracellular Na^+ concentrations, 34 and 33 mEq/kg. cell water, respectively.

The question was raised by several groups of investigators in recent years as to whether (Na^+-K^+)-activated ATPase and the transmembranous movements of sodium and potassium ions were associated with metabolic processes within the cell. Quastel and Kaplan (23 - 25) have looked at the normal human small lymphocyte in particular. They found that phytohemagglutinin (PHA), a potent stimulator of blast transformation and mitosis of DNA, RNA, and protein synthesis when applied to lymphocytes in culture, produced a significant increase in the uptake of $^{42}K^+$. Ouabain at concentrations greater than 10^{-8} M. added to the lymphocyte culture 24 hours prior to assay prevented the PHA-induced increase in DNA, RNA, and protein synthesis and $^{42}K^+$ -uptake. Quastel and Kaplan postulated that the stimulation of blast transformation involved initially an increment in the intracellular potassium "level" (25) (not concentration). This increment, according to the authors, originated from an enhancement of plasma-membrane (Na^+-K^+)-activated ATPase activity. Ouabain, a known inhibitor of this enzyme, prevented PHA from initiating blastogenesis. They implied that both PHA and ouabain acted at the cell membrane to produce their effects. Unfortunately, their papers revealed some apparent inconsistencies. First of all, they did not account for the increased rate of $^{42}K^+$ efflux,

primarily a passive process, as well as influx after PHA treatment. Secondly, the investigators presented a set of experiments showing that a 6.2×10^{-7}M. ouabain had no significant effect on the rate of K^+ efflux in lymphocytes that had been pre-loaded with $^{42}K^+$ but without exposure to PHA. However, they failed to present experimental evidence for the inhibition of $^{42}K^+$ influx by ouabain at that particular concentration. If indeed ouabain at this concentration were inhibiting membrane (Na^+-K^+)-activated ATPase, ouabain, of course, would not have been expected to have had any effect on $^{42}K^+$ efflux since this is a passive, non-ATP-dependent process. In addition, they pointed out that 24-hour ouabain treatment <u>in vitro</u> caused alteration of nuclear morphology and that PHA caused increased endocytosis. It may be that they were dealing with an effect of ouabain which is independent of a specific effect on active K^+ transport. As stated in their communications, the steroid moiety of ouabain might be the culprit here even though they were unable to mimic the same effects with corticosteroids substituted for ouabain at similar concentrations.

Since ouabain possesses a steroid moiety and since glucocorticoids have long been known to be potent lymphocytolytic agents, it is not surprising that many groups of investigators have studied

the effect of cortisol (hydrocortisone) on various metabolic pathways in lymphocytes. Most of these studies have been performed on rat thymic lymphocytes (thymocytes). In 1960, Hechter and Lester (26) presented a comprehensive analysis of the early studies of the effect of various hormones, including the corticosteroids, on glucose transport, amino acid transport, and other permeability phenomena. More recently, several groups (27 - 37) have been investigating the effect of cortisol on cellular morphology, on protein, RNA, and DNA synthesis, and glucose and amino acid uptake in rat thymocytes. Makman et al. (33) in 1966 reported that cortisol in concentrations of from 10^{-10} to 10^{-5} M. (10^{-7} M. being the physiologic concentration) inhibited in vitro DNA, RNA, and protein synthesis in rat thymocytes. In addition, a good correlation was obtained with in vivo studies. In 1968, the same investigators (34) demonstrated that in vitro treatment of rat thymic lymphocytes with 10^{-6} M. cortisol for three hours significantly reduced the uptake of ^{14}C-AIB (α-aminoisobutyric acid, a synthetic, nonmetabolizable amino acid). Two years later, Makman et al. (36) discovered that 10^{-6} M. hydrocortisone inhibited DNA-dependent RNA polymerase activity in vitro in rat thymocyte nuclei. Stuart and Ingram (37) demonstrated that 10^{-6} M. cortisol significantly inhibited glucose uptake in rat thymocytes incubated aerobically and anaerobically.

More recently, Makman and his associates (35) concluded after "at least two experiments" that treatment of rat thymocytes in cell suspension with 10^{-6} M. cortisol *in vitro* inhibited total rubidium-86 uptake by roughly 20%. This conclusion was based on a small number of experiments and the incubation media consisted of a saline solution containing only 1 mM KCl. They were presumably dealing with a low potassium concentration and competition between potassium and rubidium ions. It would have been more appropriate if the media had included either 5 mM RbCl and tracer amounts of $^{86}Rb^{+}$ or 5 mM KCl and $^{42}K^{+}$, although rubidium usually behaves in a fashion very similar to potassium (3). In any case, Makman and his coworkers are currently operating on the hypothesis presented in 1967 (38) that cortisol is some way stimulates the production of a new protein which in turn produces all the effects discussed above since they found various inhibitors, such as cycloheximide and actinomycin D, prevented the action of cortisol in a number of combination and time-course studies (31, 32, 35, 36).

With the foregoing as a backdrop, the studies that follow were performed to determine whether a Na-K pump existed in lymphocytes. This was assessed not by enzymatic analysis but by inference from radiosodium- and radiopotassium- uptake studies in the presence and absence of ouabain and oligomycin. At the same

time, an attempt was made to compare cation flux rates in rat thymic lymphocytes and human chronic lymphatic leukemic (CLL) and normal non-leukemic (NNL) tonsillar lymphocytes to determine if there were a difference in membrane permeability and/or ouabain-inhibitable (active) transport rates between rat thymocytes and human lymphocytes (a species difference) and between human CLL and NLL lymphocytes (a neoplastic difference). Intracellular cation concentration and cell volumes were also examined. In addition, experiments were performed to test the validity of the conclusions of Makman et al. (35) that cortisol in vitro reduced monovalent cation influx in rat thymocytes.

METHODS

Preparation of Rat Thymocytes

125 - 160-gram male Sprague-Dawley rats (Chordata Corp., Ontario, New York) were decapitated, bled, and thymectomized using relatively bloodless, blunt dissection after opening the thorax. The thymus glands were dissected free of connective tissue in a glass Petri dish on ice filled with ice-cold buffer (5 mM KCl, 135 mM NaCl, 1.25mM $CaCl_2$, 1.00 mM $MgCl_2$, 10 mM tris (hydroxymethyl) aminomethane, pH adjusted to 7.4 with 6 N HCl). Surface blood was rinsed free with ice-cold buffer. Phase-contrast microscopic study of final cell suspensions revealed less than one per cent red cells. The two lobes of each thymus were separated and minced with fine dissection schissors. A quantity of tissue not exceeding the equivalent of three whole glands and two milliliters of buffer were placed into an ice-cold 13-ml. Dounce homogenizer and dispersed gently using a loose-fitting pestle, stroking a maximum of 6 times. Care was taken to minimize mechanical trauma to the thymocytes. In order to remove large pieces of tissue, the dispersed cells were poured through four layers of 2" x 2" Johnson & Johnson gauze sponges into an ice-cold 50-ml. glass beaker. The filtered cell suspension was poured into a sterile 10-cc plastic syringe fitted with a

stainless-steel Millipore support screen and filtered into a 15-ml. plastic centrifuge tube. The filtrate was centrifuged at 200G for 5 minutes using the Sorvall refrigerated centrifuge (HG-4 head). After decanting the supernatant, the cells were resuspended gently in a predetermined amount of the appropriate buffer (same as the aforementioned buffer except for the addition of 100 mg% of D-glucose)*.

Preparation of Human Chronic Lymphatic Leukemic (CLL) Lymphocytes

The patients selected for study were male and female adults over the age of 40 years with chronic lymphatic leukemic (CLL) on the basis of (a) longstanding white cell counts greater than 20,000 cells per microliter of which 95 to 99% were small lymphocytes (b) marrow lymphocytosis, (c) often an associated thrombocytopenia and/or anemia, and (d) splenomegaly and lymphadenopathy. All were untreated at the time of study. The patient's blood was collected in 10-cc Vacutainer tubes with 143 U.S.P. units of sodium heparin per tube. Within 24 hours of collection, one-half of the

*Because of the need for a high specific activity of ^{42}K ($t_{\frac{1}{2}}$ = 12.36 hours) in the K^+-influx experiments, an initial buffer K^+ concentration of 4.8 mM was occasionally used so that the final calculated K^+ concentration after the addition of ^{42}K ranged from 4.92 to 5.15 mM.

volume of whole blood was centrifuged in the Sorvall refrigerated centrifuge at 900 G for 10 minutes and the buffy coats removed with Pasteur pipettes and mixed with the remaining volume of whole blood. A mixture of 4 parts leukocyte-enriched whole blood and 1 part sterile 4.4% (w/v) polyvinylpyrrolidone (PVP) in buffer (same as above minus D-glucose) was allowed to undergo sedimentation for 60 to 90 minutes (depending upon the intrinsic ESR) at room temperature. The plasma-leukocyte layer was removed with a Pasteur pipette and centrifuged at 200 G in the Sorvall refrigerated centrifuge for 5 minutes. After decanting the supernatant, the procedure of cell preparation was as for rat thymocytes. Less than 10% (usually less than 3%) of cells were erythrocytes as revealed by differential cell counts by phase-contrast microscopy.

Preparation of Human Normal Non-Leukemic (NNL) Lymphocytes

Human normal non-leukemic lymphocytes were prepared from adenoids and palatine tonsils removed surgically from pediatric patients under the age of 10 years on the Ear, Nose & Throat Service at the University of Rochester Medical Center. The operative procedures were indicated for recurrent otitis media and/or for habitual mouth-breathing and were performed at a time when the patient's condition had stabilized and the patient was free of any apparent infection. Immediately following removal, the lymphoid tissue was placed into isotonic saline in a plastic

snap-cap container. Within 5 minutes it was placed on ice. Five to 10 minutes thereafter the tissue was removed from saline and placed into a Petri dish containing ice-cold buffer. Obviously hemorrhagic specimens were discarded. As discussed earlier in the preparation of rat thymocytes, the specimens were stripped of connective tissue and rinsed well in buffer transferring the pieces to one or two other buffer-containing Petri dishes depending upon the amount of apparent erythrocyte contamination. The rest of the procedure followed that for the preparation of rat thymocytes. This smears stained with Wright's stain were prepared for differential cell counts after centrifuging 0.3 cc of a cell suspension at 900 G for 10 minutes, aspirating the supernatant, and resuspending the cells in about 0.2 cc of human type A- serum (A- was used for convenience). At most, 10% erythrocyte and 2% epithelial-cell contamination was revealed by phase-microscopic examination of wet preparations.

Cell Concentration Range

The final cell concentration for rat thymocytes in the individual experiments ranged from 0.875 to 3.98 x 10^8 cells/ml. Because of the greater absolute volume of cell water per cell, the range of cell concentration for human CLL lymphocytes was somewhat smaller, being 0.6 to 1.4 x 10^8 cells/ml. and that for human NNL lymphocytes being 2.5 to 4.9 x 10^7 cells/ml.

Volume, Cell Counts, Cation and Water Content

The relative volume and volume distribution of erythrocytes and lymphocytes were determined on a Nuclear Data particle-volume analyzer with a Coulter-counter 48-micron-diameter orifice. Absolute cell volume in cubic microns was measured by carrying out simultaneous cell counts and packed cell volume measurements using thrombocytocrit tubes (Arthur H. Thomas Co., Philadelphia, Pa.).

Lymphocyte cell counts were made in duplicate using a Bright Line hemocytometer and phase-contrast microscope.

To determine lymphocyte cation and water content aliquots of cell suspension pre-incubated as described in the following section were placed into weighed tubes. The samples were centrifuged as below and the supernatant was poured off and aspirated. The cell buttons were resuspended in 2.5 ml. of a solution containing 150 mM choline chloride, 10 mM tris (hydroxymethyl) aminomethane, 5 mM non-radioactive sucrose, ^{14}C-sucrose, adjusted to pH 7.4 with concentrated HCl. The resuspended samples were centrifuged for 5 minutes at 200 G. The supernatant was decanted and the cell buttons were weighed when the tubes cooled to room temperature. Duplicate and triplicate samples were run when there was an adequate quantity of lymphocytes. Each cell button was digested with 0.05 ml. of analytical-grade

concentrated nitric acid and placed into the water-bath at 37° C. for 30 minutes. 0.95 ml. of distilled de-ionized water was added to the digests and centrifuged at 1500 G for five minutes in the Sorvall table-top centrifuge. The supernatant was poured off and saved. 1.0 ml. of distilled de-ionized water was added to the sediment and centrifuged at 1500 G for five minutes. The supernates were decanted and added to the corresponding tubes of already saved supernates. After each supernate sample was mixed on a Vortex mixer, a 0.05-ml. sample was taken from each for liquid-scintillation counting. Quenching was corrected for by running a nitric-acid blank in parallel and taking a 0.05-ml. sample as above. Cell water was calculated as cell net weight (total wet weight minus ^{14}C-sucrose space) minus cell dry weight. The remainder of the supernates were poured into 10-ml. volumetric flasks containing 15mM lithium chloride and diluted up to 10 ml. with distilled de-ionized water. The quantity of Na^+ and K^+ in the samples was measured by flame photometry using the Instrumentation Lab., Inc. Model 143 Flame Photometer with LiCl as the internal standard. Nitric-acid blanks were run with each experiment.

In Vitro Potassium Uptake

The undiluted cell suspensions in snap-cap plastic vials were incubated in a water-bath at 37° C. with the shaker set at 100 r.p.m. for 30 minutes in order to permit the cells to attain steady-state conditions in their new extracellular environment. Also incubated at the same time were the necessary amounts of aforementioned buffer with 100 mg% glucose, with and without enough ouabain to give a final concentration of 5×10^{-3} M., and enough $^{42}K^{+}$ to yield a medium specific activity of 0.8 to 2.0 x 10^{7} cpm/millimole K^{+}.

After 60 minutes of pre-incubation, the appropriate equal volumes of pre-incubated cell suspension and pre-warmed buffer with $^{42}K^{+}$, with and without ouabain, were combined. The control and ouabain-treated cell suspensions were incubated in plastic snap-cap vials at 37° C. with the shaker set at 100 r.p.m. A single 1.0-ml. aliquot was taken from each vial using sterile disposable 1.0-ml. glass pipettes immediately after mixing $^{42}K^{+}$-labelled buffer with the cell suspensions ("zero time") and placed into tared 10mm x 75mm disposable glass culture tubes (Kimble) packed in ice. Sixty seconds elapsed between "zero time" and the time the suspensions reached 2° C. Single 1.0-ml. aliquots were also taken at 7', 15', 22', and 30' (or 8', 16',

23', and 31' in early experiments). For each time period, the samples were immediately centrifuged using the Sorvall refrigerated centrifuge by turning the "r.p.m." dial to the end of the scale (5000), switching off the motor when the head speed reached 1740 G, and manually slowing the head from 200 to 300 G. This procedure was found to give adequate separation with minimal mechanical trauma to the cells and with minimal elapsed time between sampling and separation of the cells from ^{42}K in the medium. The supernatant was aspirated, only the "one-minute" supernatant was saved for specific-activity determination. The cell buttons were resuspended at 2° C. in 2.5 ml. of the original non-radioactive buffer minus the additional dextrose. The suspensions were centrifuged as before. The supernatants were decanted, aspirated, and discarded.

The washed cell buttons and supernatants were counted using the Radiation Instrument Development Laboratory Model 200-S well-type γ-counter set at 1350 volts. γ emissions from each cell button and 0.02-cc sample of supernatant were counted for two minutes each. Counting for each individual experiment was completed within 30 minutes so as to obviate correcting for isotopic decay (approximately a 2% reduction in specific activity in 30 minutes).

Cell buttons were placed in a drying oven at 80 to 90° C. overnight and tare weights were performed the following day when the tubes were at room temperature.

Isotope distribution ratios and influx rates were calculated as described below under CALCULATIONS.

In Vitro Sodium Uptake

Sodium uptake was determined in the same way as the potassium uptake except for the following additions or deletions. Enough $^{22}Na^{+}$ was added to the buffers to give specific activities ranging from 1.26 to 2.07 x 10^{6} cpm/mmole Na^{+}. Each cell button and 0.02-cc sample of supernatant were counted for five minutes each as the decay-factor presented no problem.

In Vivo Distribution of ^{42}K and ^{24}Na in Rat Thymus

Four 130-gram male Sprague-Dawley rats were injected intraperitoneally with 1.0 ml. of sterile isotonic saline containing approximately 20 microcuries of $^{42}K_2CO_3$ and placed into cages without food or water. After 30 minutes and three hours, two rats were anesthetized with ether. Blood was withdrawn from the heart, allowed to clot, and the serum removed after centrifuging at 900 G for 10 minutes. Following manual separation of the cervical vertebrae to effect the death of the animal, the thymus

gland was removed, washed with ice-cold buffer, blotted dry, stripped of connective tissue, and placed into tared glass tubes. The glands were weighed, dried overnight at 90° C., and reweighed. Glands and an aliquot of the corresponding serum were counted for five minutes each. One week later the serum was analyzed for Na^+ and K^+ content using the factor of 92% water by weight for rat serum as previously determined by analyzing the water content of serum samples from three different rats.

For the ^{24}Na distribution study, three 200-gram male rats of the Sprague-Dawley strain were injected intraperitoneally with 1.0 ml. of sterile isotonic saline with approximately 20 microcuries of $^{24}Na_2CO_3$. One rat was sacrificed after three hours and two after 24 hours. The rats were provided with food and water. Loss of $^{24}Na^+$ in urine and feces was not accounted for. The remainder of the procedure followed that for the *in vivo* ^{42}K study.

Effect of Oligomycin on ^{42}K Uptake

In all three lymphocyte types, at least one experiment was performed to determine if oligomycin at a final concentration of 500 µg/ml. *in vitro* had an effect on ^{42}K uptake in these cells. The procedure was identical to that for determining K^+-influx rates except for the addition of a quantity of dextrose-containing buffer without ouabain but with oligomycin to an equal volume of

cell suspension placed in a third vial.

Effect of Cortisol and/or Cycloheximide on ^{42}K Uptake in Rat Thymocytes

To determine whether hydrocortisone (cortisol) and/or cycloheximide had any effect on ^{42}K uptake in rat thymocytes, cell suspensions in plastic snap-cap vials at a concentration of 8×10^7 cell/ml. were incubated at 37° C. for three hours without drug and with 1.0×10^{-5} M. hydrocortisone and/or a 4.0×10^{-4} M. solution of 88.3% "active" cycloheximide. After three hours, buffer with ^{42}K with or without ouabain (5×10^{-3} M.) and with or without the respective drugs at identical concentrations was added to the respective vials. The suspensions (cell concentration of 4×10^7 cells/ml.) were incubated for 10 minutes and 1.0-cc aliquots were removed in triplicate from the ouabain- and non-ouabain-containing vials. Samples were handled as presented above under the K^+-uptake procedure. Five experiments were performed with or without the addition of cortisol alone. Two experiments were done with no drug, hydrocortisone alone, cycloheximide alone, and hydrocortisone plus cycloheximide. Triplicate samples and 0.02 ml. of their corresponding pooled supernatant were counted for two minutes each as before within a 10-minute period so that no correction for isotopic decay was necessary.

MATERIALS

Radioisotopes

^{42}K and ^{24}Na as the carbonate salt were supplied by International Chemical and Nuclear Corp. (ICN), Chemical and Radioisotope Division, Irvine, Calif. The concentration of $^{42}K^{+}$ added did not exceed 0.35 mM and the carbonate did not lead to alteration of medium pH. ^{42}K and ^{22}Na as the chloride salt were supplied by Cambridge Nuclear Corp., Billerica, Mass. D-sucrose-^{14}C was provided by New England Nuclear Corp., Boston, Mass.

Chemical Agents

Ouabain (octahydrate - Strophanthin-G) was obtained from Sigma Chemical Co. as was oligomycin (15% A and 85% B). Both were dissolved in buffer after warming for one hour at 37° C.

Hydrocortisone (cortisol) was purchased from Calbiochem. 0.0045 g of cortisol was placed into 50 ml. of buffer and warmed at 37° C. for one hour. Predetermined aliquots were then placed into the cell suspension and buffer to maintain a concentration of 1.0×10^{-5} M.

Cycloheximide (Actidione, U-4527) was supplied by Upjohn with a potency rating of 883 mcg./mg.

CALCULATIONS

Distribution Ratios

The calculation of the radioisotope distribution ratios were based upon the following formula:

$$\frac{\text{Radiocation}_i}{\text{Radiocation}_o} \text{ (Ratio)} = \frac{\text{cpm}_i/\text{kg cell water}}{\text{cpm}_o/\text{kg medium water}}$$

This is equivalent to:

$$\text{Ratio} = \frac{\dfrac{P'_c(t) - C_t(t)}{0.001 * D_w * (W/(1.0 - W)}}{\dfrac{P_{tot} - P'_c(t) + C_t(t) * 10^3}{(1.0 - \alpha)}}$$

Where:

- $*$ = signifies a multiplication in Fortran computer language
- $P'_c(t)$ = pellet cpm at time t
- $C_t(t)$ = extracellular cpm present in pellet ("trapped" radioisotopic cation)
- D_w = dry weight of pellet in grams
- W = proportion of water content per cell
- P_{tot} = total cpm per 1-ml. cell suspension

  ($P_{tot} = P_s(t) + P_c(t) = P_s(o)$ where

$P_s(t)$ = actual cpm in supernatant of a 1-ml. aliquot

$P_c(t)$ = actual cpm in pellet of a 1-ml. aliquot)

α = cytocrit (proportion of cells in 1-ml. aliquot of cell suspension by volume).

For each experiment, the cell concentration, the dry weight of each pellet, the cpm/ml of the supernatant of the first time period for each set of samples ("total", "ouabain-insensitive", etc.) and the pellet counts-per-minute readings were all determined. The activity of the supernatant was redetermined by computer (IBM 360 Model 50) for each time period from the first sample from the following formulae:

$$P_s(1) = (P'_s(1)/0.968) * (1.0 - \alpha)$$

$$P_s(t) = P_s(1) - (P'_c(t) - P'_c(1))$$

where: $P'_s(1)$ = observed cpm/ml of 1st time interval supernatant

0.968 = correction factor experimentally determined to adjust for isotopic activity lost in wash.

$P_s(1)$ = actual cpm of supernatant

C_t was determined from the following equation:

$$C_t(t) = P_s(t) * 0.40 * G * 0.032$$

where: 0.40 = factor correcting for a 2.5-ml. wash volume

and 0.032 = proportion of actual supernatant cpm found in wash of 2.5-ml. volume

G = volume in milliliters of trapped extracellular water in pellet for given dry weight. This parameter varies for each cell type (see below)

The cytocrit (α) was determined from an equivalency of number of cells of a mean volume per 0.02 value for α as shown below.

All of the parameters determined for each of the three cell types are tabulated below.

Influx Rates

The formulae and computer program (in Fortran IV Computer language) was developed by Dr. David Goldstein, Dept. of Radiation Biology and Biophysics, University of Rochester, Rochester, New York. Only the basic equations are discussed here.

Let

P_c = cpm in cell pellet in 1-ml. suspension

P_s = cpm in supernatant in 1-ml. suspension

α = cytocrit

N = number of cells/ml. suspension

V_c = volume in cm^3/ cell

A_c = surface area in cm^2/cell calculated from V_c assuming the cell to be a sphere

K_c = mEq K^+ in cell pellet per 1-ml. suspension

K_s = mEq K^+ in supernatant of 1-ml. suspension

ϕ_{sc} = flux of radioisotope from the medium to the cells (influx)

ϕ_{cs} = flux of radioisotope from the cells into the medium (efflux)

p^*_s = specific activity of cation within the supernatant

p^*_c = specific activity of cation within the cells

$P_{tot} = P_s + P_c$

$K_{tot} = K_s + K_c$

Then, for the steady-state, where the intracellular concentrations of K^+ and Na^+ are maintained, the following model was used:

$$\frac{dP_c}{dt} = NA_c \left(\phi_{sc}\, p^*_s - \phi_{cs}\, p^*_c\right)$$

Since no volume change was observed experimentally during the period of time over which these experiments were performed,

we can reasonably set

$$N = \alpha/V_c$$

This gives us:

$$\frac{dP_c}{dT} = \alpha\left(\frac{A_c}{V_c}\right)\{\phi_{sc}p_s^* - \phi_{cs}\,p_c^*\}.$$

In the non-steady state, as is the case with the ouabain-treated cells, where intracellular Na^+ and K^+ cation levels are changing at each instant in time, it was assumed that intracellular Na^+ and K^+ concentrations were changing in a linear fashion over the 30-minute period of incubation with the radioisotope. Hence, $K_c(t) = K_c(o) - \lambda t$.

In the steady state, then, $\lambda = o$, whereas in the non-steady state λ represents the slope of the decreasing intracellular K^+ concentration.

In order to simplify calculations, the ratio of cell surface area (A_c) to cell volume (V_c) was assumed to be constant. (V_c was shown to be constant by experimental observations). Thus, since

$$(\phi_{sc} - \phi_{cs})\ N \cdot A_c = \frac{dK_c(t)}{dT}\ ,\ (\phi_{sc} - \phi_{cs})\ \frac{\alpha A_c}{V_c} = -\lambda$$

which becomes: $\phi_{sc} - \phi_{cs} = \frac{-\lambda V_c}{\alpha A_c}$

Substituting and rearranging the variables, we obtain:

$$\frac{dP_c}{dt} = \alpha\left(\frac{A_c}{V_c}\right)\left\{\phi_{sc}\frac{(P_{tot}-P_c)}{(K_{tot}-K_c)} - \left[\phi_{sc} + \frac{\lambda V_c}{\alpha A_c}\right]\frac{P_c}{K_c}\right\}$$

$$= \alpha\left(\frac{A_c}{V_c}\right)\left\{\phi_{sc}\left[\frac{K_c P_{tot} - K_{tot} P_c}{K_c (K_{tot}-K_c)}\right] - \frac{\lambda V_c}{\alpha A_c} \cdot \frac{P_c}{K_c}\right\}$$

Although such an assumption may well not be correct, it was assumed that ϕ_{sc}, the influx of cation, was constant during the experiment. Then using a least-squares non-linear regression-analysis subroutine, a computer program was written to determine the value of ϕ_{sc} that would best fit the data. Preliminary estimates of ϕ_{sc} of from 10^{-9} to 10^{-8} mEq/cm^2/min. were used.

The parameters α, A_c, V_c, λ_{Na}, λ_K, and others which differ for each of the three groups of lymphocytes dealt with in these experiments are tabulated below:

	RAT THYMOCYTES	HUMAN CLL LYMPHOCYTES	HUMAN NNL LYMPHOCYTES
G	$4.61 \cdot D_w + 0.02^{\Delta}$	$2.45 \cdot D_w + 0.02^{\Delta}$	$2.16 \cdot D_w + 0.02^{\Delta}$
α	$(F/1.40)\cdot(0.02)$	$(F/0.975)\cdot(0.02)$	$(F/0.68)\cdot(0.02)$
A_c (in cm^2) ††	1.319×10^{-6}	1.682×10^{-6}	2.13×10^{-6}
V_c (in cm^3) †	1.426×10^{-10}	2.0517×10^{-10}	2.94×10^{-10}
$K_c(o)$ (in mEq) †	0.143	0.139	
$Na_c(o)$ (in mEq) †	0.029	0.026	
λ'_K * (in mEq/liter/min)	0.00143	0.000635	
λ'_{Na} *	-0.00145	-0.00055	
W	0.80	0.78	0.78

NOTE: F = number of cells x 10^8 per cm^3

† V_c, $K_c(o)$, $Na_c(o)$ represent mean values as calculated from at least five separate observations.

†† A_c was derived from the cell volumes after assuming that the lymphocyte is a smooth sphere, i.e. $A_c = 4\pi(\frac{3V_c}{4\pi})^{2/3}$.

* Listed are the λ' 's for the non-steady state, i.e. when the cells were treated with ouabain. For the total influx under steady-state conditions, λ'_K and $\lambda'_{Na} = 0$. λ (non-primed) as in the equations above = $\lambda' \cdot W \cdot \alpha$ in each case.

$^{\Delta}$ Trapped extracellular water volume was measured repeatedly over a wide range of pellet dry weights in all three groups of lymphocytes. For these determinations ^{14}C-sucrose tracer in a solution made up of 150 mM choline chloride, 10 mM Tris, 5 mM nonradioactive sucrose, buffered to pH 7.4 with concentrated HCl, was used as the wash solution. The amount of decay from ^{14}C in the trapped extracellular water was measured with the Nuclear Chicago liquid-scintillation counter. The dry weights (in grams) were plotted against the trapped water volume (in mls). The best-fitting straight line was determined by least-squares linear-regression analysis. As seen here, the slope of this line decreases with the increasing volume. The Y-intercept of 0.02 was a constant observation and probably represents the quantity of wash left on the inner surface of the tubes into which the aliquots of cell suspension were placed.

RESULTS

Cell Volume Distribution and Morphology

The volume distribution curves of each group of lymphocytes were unimodal (Figure 1). The second small peak in the erythrocyte curve is considered to be electrical artifact. Both rat thymic and human CLL lymphocyte cell volumes appeared to be approximately normally distributed about the mean. On the other hand, human normal non-leukemic lymphocytes apparently included a sizable population of much larger cells, accounting for the slow tailing off of the distribution curve from a modal peak lying to the left of that for human CLL lymphocytes. These findings correlated well with the thrombocytocrit-tube cell-volume determinations. Rat thymocytes had a mean cell volume (MCV) of 142.6 μ^3 with a standard deviation (S.D.) of ± 15.85 μ^3, human CLL lymphocytes a MCV of 205.17 μ^3 ± a S.D. of 13.15 μ^3, and human tonsillar lymphocytes a MCV of 294 μ^3 with a S.D. of ± 31.28 μ^3. A greater appreciation of the disparity of cell sizes as well as the degree of morphologic integrity preserved in isolation of the cells was obtained from microscopic studies (Figure 2). A small proportion of blastic-looking cells in the tonsillar lymphocyte preparations was observed (as in Figure 2C).

PARTICLE FREQUENCY
CHANNEL NUMBER

In addition, phase-contrast microscopy of lympocyte suspensions performed up to four hours after the onset of incubation with and without ouabain revealed no loss of morphologic integrity.

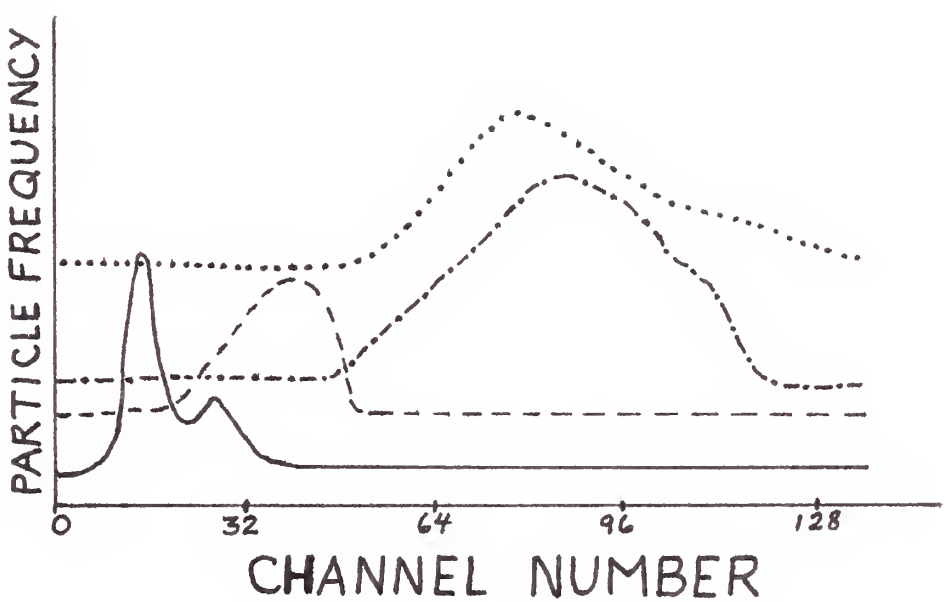
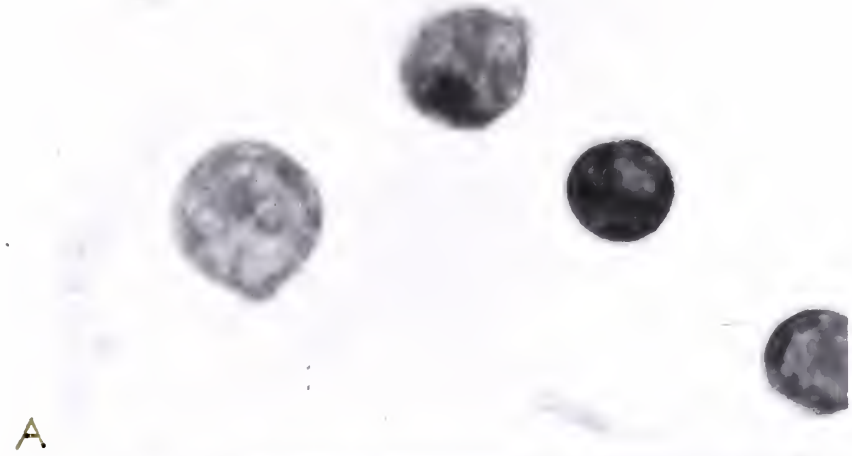


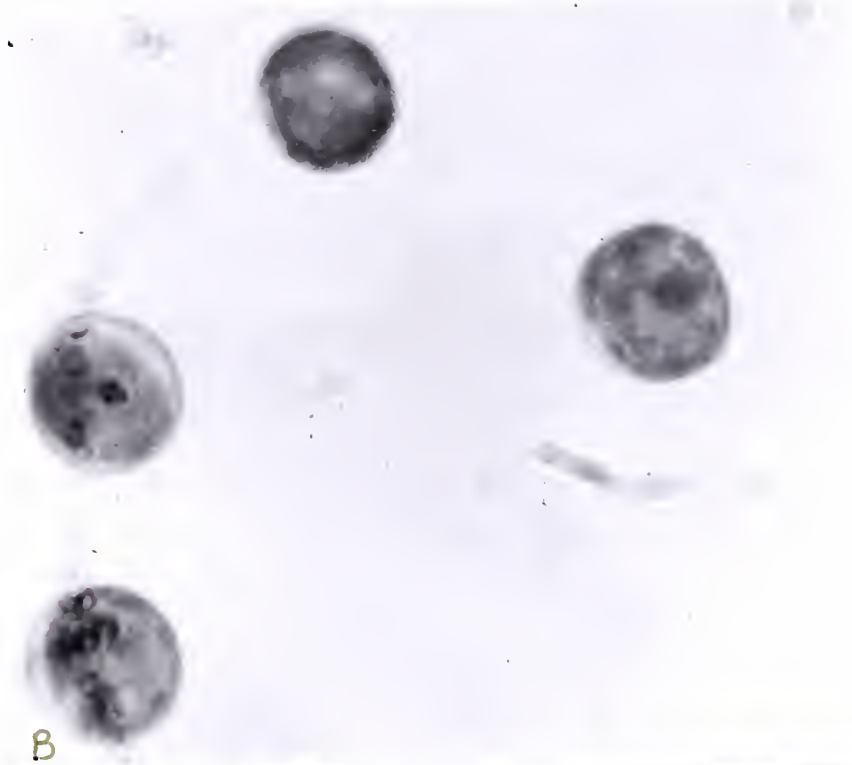
Figure 1. The volume distribution of human erythrocytes, rat thymocytes, human CLL lymphocytes, and human normal non-leukemic lymphocytes as traced from Polaroid photographs of the oscilloscope read-outs from a 512-channel analyzer.

Key: _____ human erythrocytes
------ rat thymocytes
-·-·-· human CLL lymphocytes
..... human NNL lymphocytes

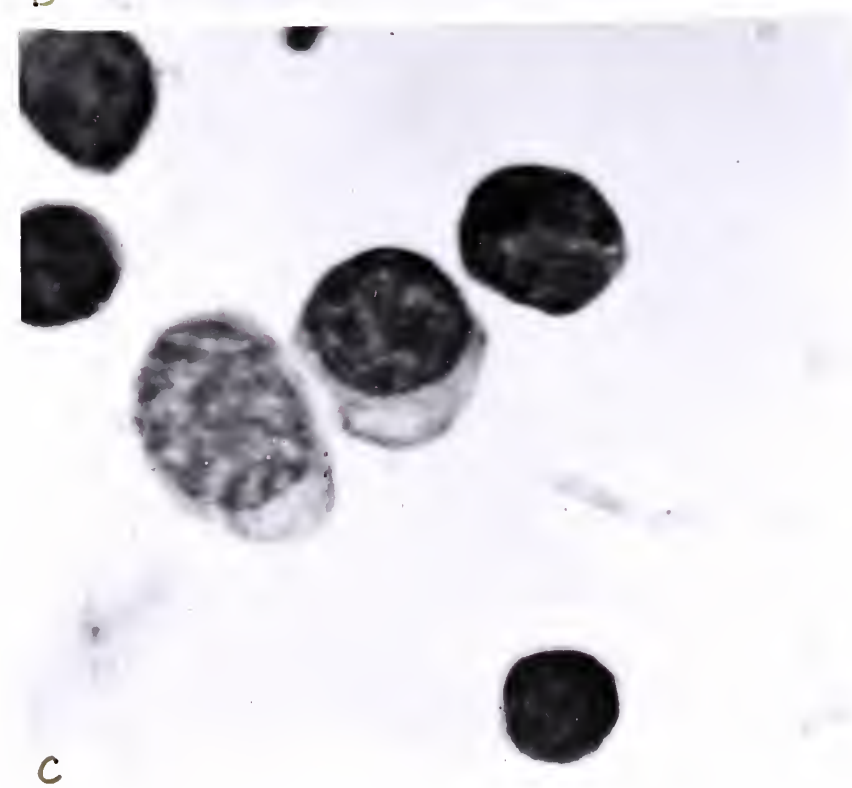
Figure 2. The morphologic integrity of isolated A) rat thymic lymphocytes, B) human CLL lymphocytes, and C) human tonsillar lymphocytes. (Original magnification 1000X). These light micrographs represent Wright-stained smears of lymphocytes suspended in autologous plasma.



A.



B



C

To determine if there were an alteration in cell volume during the period of time in which the lymphocytes were incubated with and without ouabain, several studies were performed. The volume distribution plots recorded at the onset of, during, and at the end of incubation were compared. These studies revealed no significant shift in volume distributions indicating that V_c as listed under CALCULATIONS above could be considered constant.

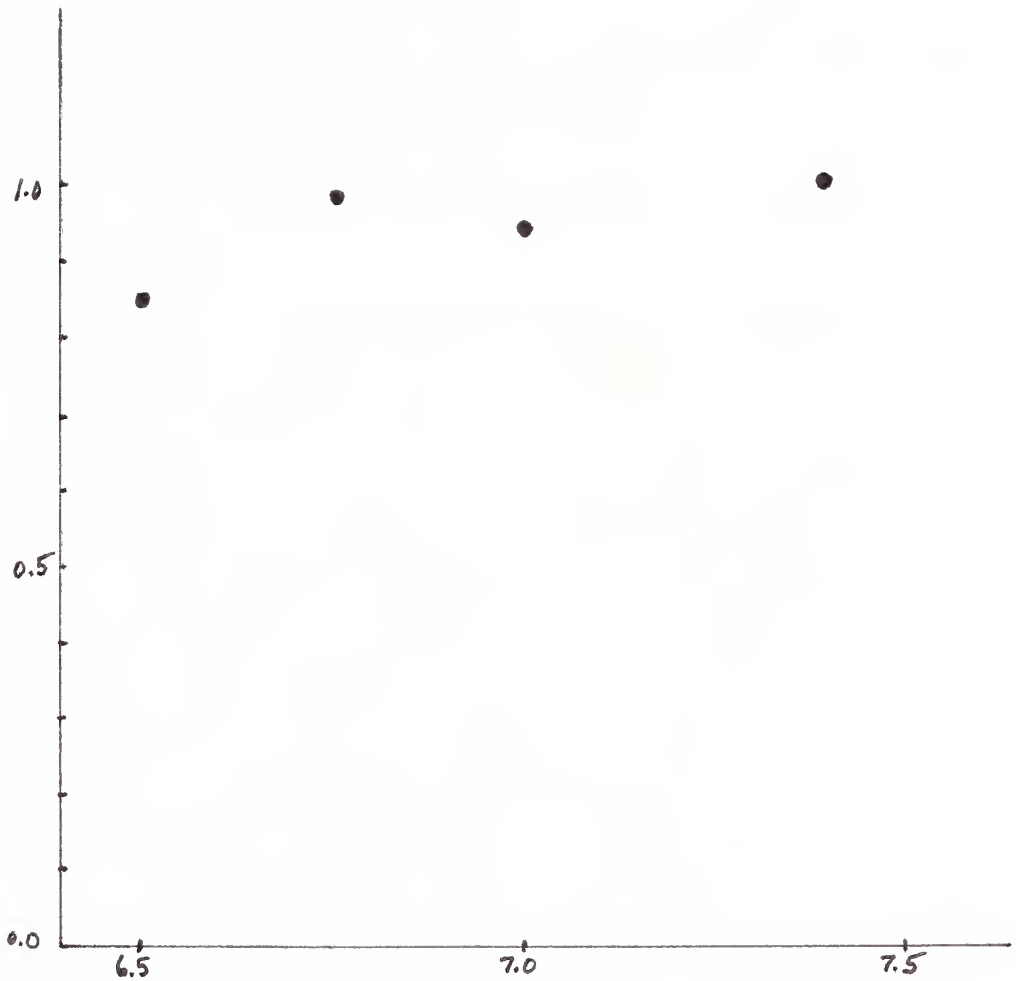
Effect of Incubation, pH, Ouabain, Temperature

The influence of several variables that might affect the measurement of cation fluxes was studied. The osmolality of the medium did not vary more than 10 milliosmoles per kilogram medium water over four hours of incubation and did not exceed 300 mOsm. During the incubation of rat thymocytes at maximum cell concentrations the pH did not drop below 6.75 and did not rise above 7.50. Under the conditions under which the *in vitro* cortisol and cycloheximide experiments were performed, the pH did not drop below 7.10. An experiment determining the effect on radiopotassium uptake of an increase in the acidity of the medium was carried out. As shown in Figure 3, there was no significant change in total K^+ uptake in rat thymocytes between pH 6.75 and 7.40. As depicted, at pH's of 6.5, a 15% reduction in K^+ influx occurred.

Figure 3. Dependence of total K^+-influx rate in rat thymocytes on medium pH.

Abscissa: pH (in pH units)
Ordinate: computer-derived K^+-influx rates in $mEq/cm^2.min$

Key: ● represents experimental point


1.0
0.5
0.0
6.5
7.0
7.5

Ouabain dose-response curves were plotted for rat thymocytes and human CLL lymphocytes as shown in Figures 4 and 5, respectively. The lowest concentration at which maximal inhibition of Na^+ efflux or K^+ influx corresponded to about 5×10^{-3} M. for both types of lymphocytes. Although no ouabain dose-response curve was plotted for tonsillar lymphocytes, the same concentration (5×10^{-3} M.) of ouabain was used in the radiocation uptake studies in these lymphocytes as well.

Thirty-minute K^+ uptake in rat thymocytes was measured after prolonged incubation (one to four hours) in vitro. No significant reduction in total, ouabain-sensitive, or ouabain-insensitive K^+ uptake was found for a three-hour period (see Figure 6). The respective computer-derived influx rates (in mEq $K^+/cm^2 \cdot min$) for one and three hours after onset of incubation were: 6.71×10^{-8} vs. 6.48×10^{-8} (total), 5.29×10^{-8} vs. 5.21×10^{-8} (ouabain-inhibitable), and 1.42×10^{-8} vs. 1.27×10^{-8} (ouabain-uninhibitable). At four hours, the rates dropped to 5.96×10^{-8}, 4.79×10^{-8}, 4.79×10^{-8}, and 1.17×10^{-8}, respectively.

In order to determine the degree of radiopotassium uptake that took place when aliquots of the cell suspension were placed at 2° C. (melting ice) as discussed under METHODS, a

Figure 4. Ouabain dose-response curve in rat thymocytes.

Abscissa: molar concentration of ouabain

Ordinate: per cent increase in intracellular Na^+ concentration after 90 minutes of exposure to ouabain *in vitro*

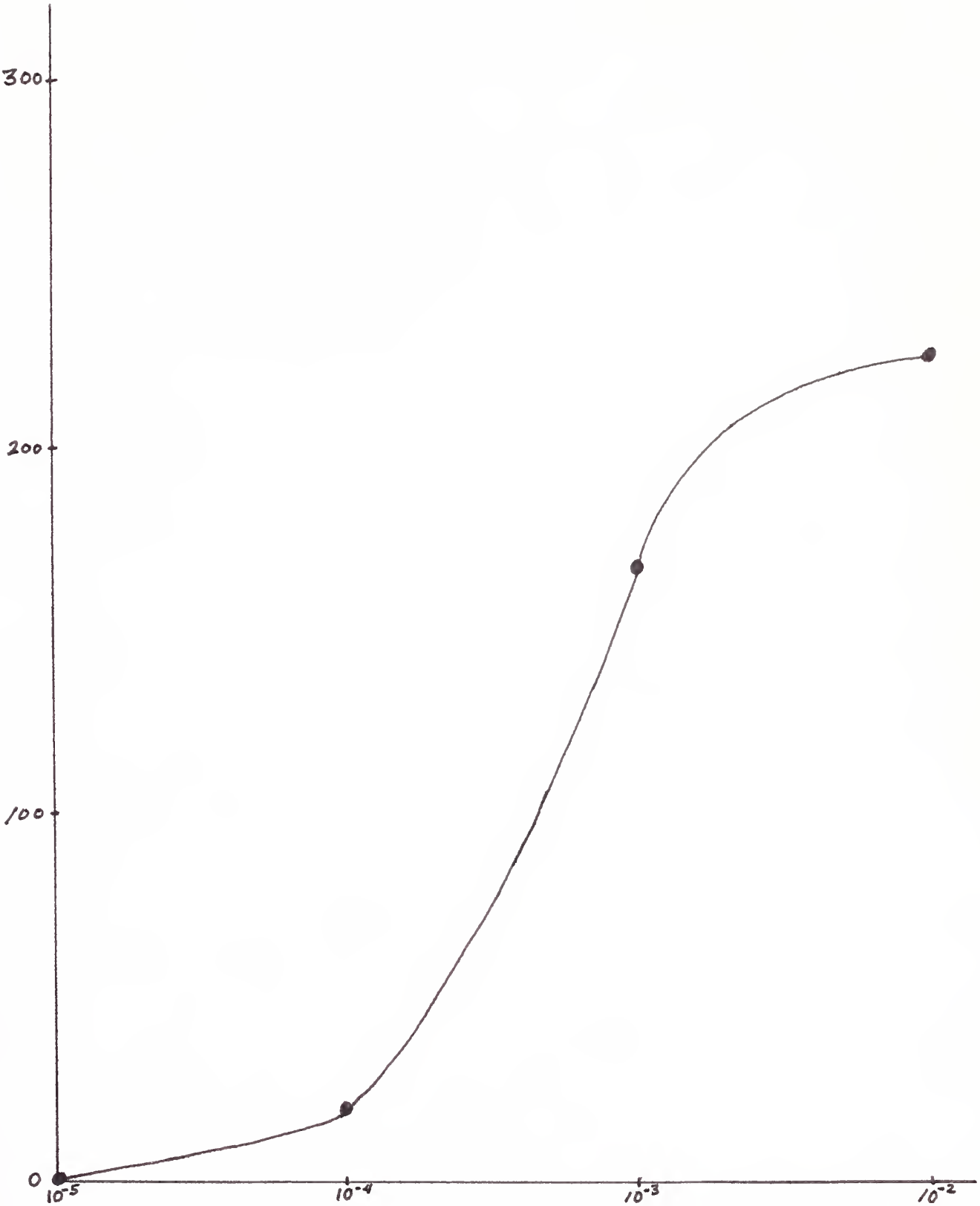

300
200
100
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}

Figure 5. Ouabain dose-response curve in human CLL lymphocytes.

Abscissa: molar concentration of ouabain

Ordinate: per cent increase in intracellular Na^+ concentration after 90 minutes of exposure to ouabain *in vitro*

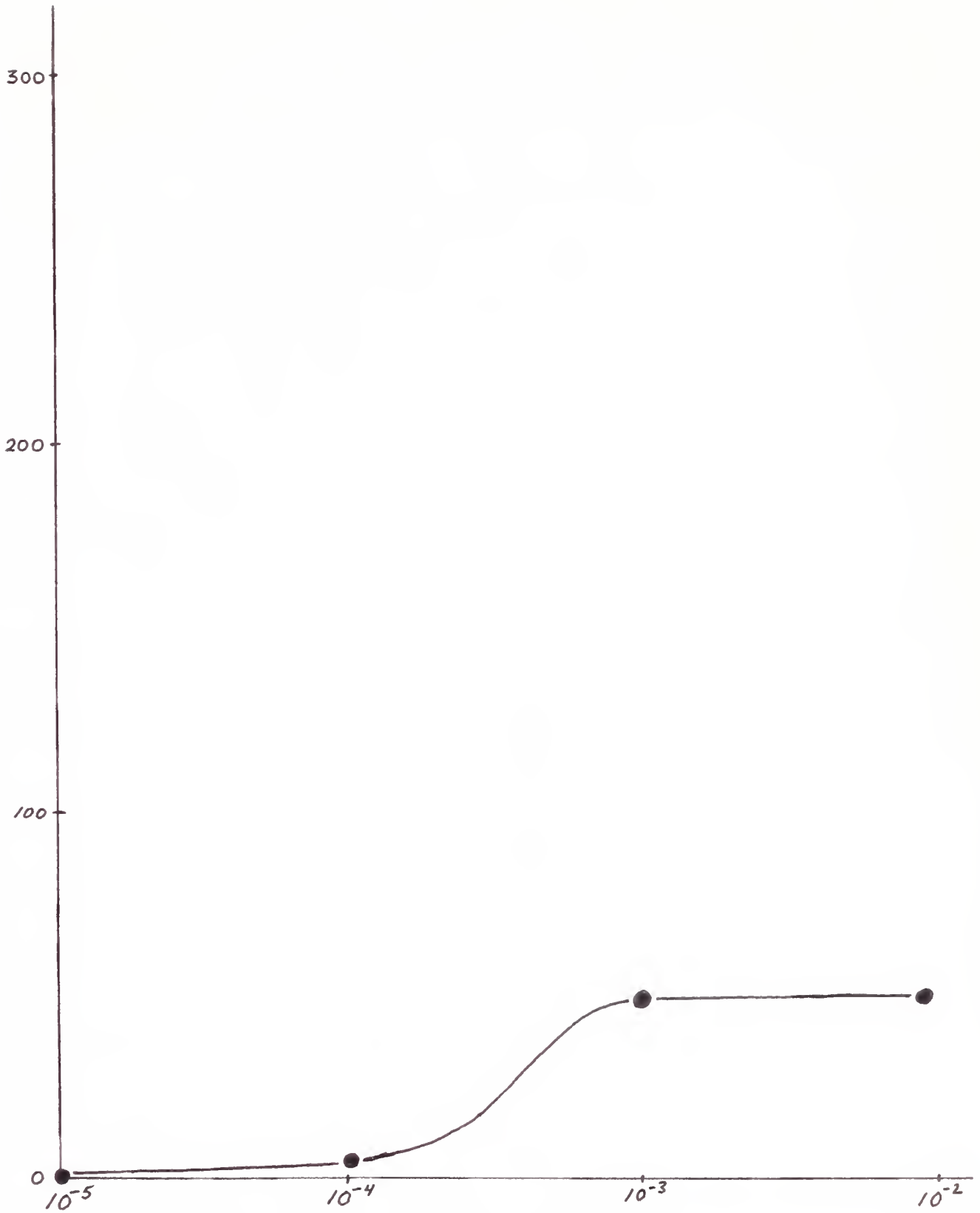
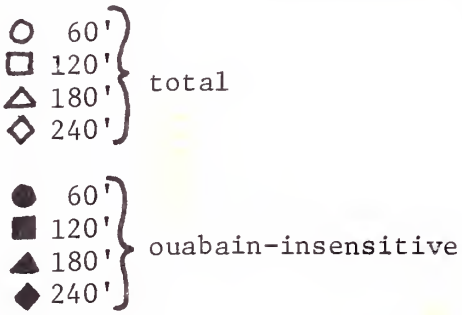

300
200
100
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}

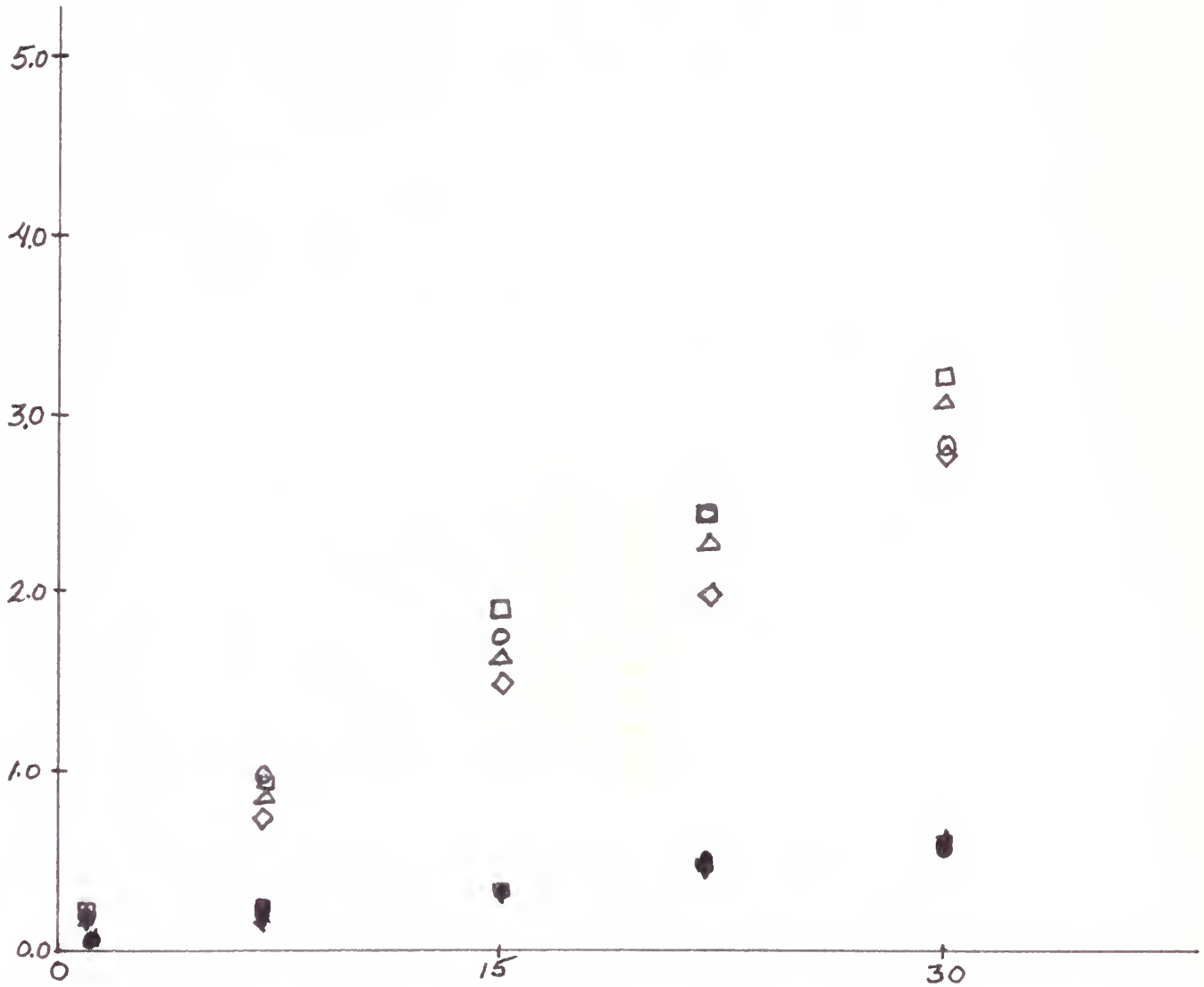
Figure 6. The effect of prolonged incubation on K^+ influx in rat thymocytes at 37° C. Isotope uptake was measured over 30 minutes.

Abscissa: time (in minutes)

Ordinate: radiopotassium distribution ratio

Key: Numbers that follow represent the length of pre-incubation prior to the addition of radioisotope.




5.0
4.0
3.0
2.0
1.0
0.0
0
15
30

thirty-minute $^{42}K^+$-uptake study with and without 5×10^{-3} M. ouabain was conducted with all the materials maintained at a temperature of 2° C. As demonstrated in Figure 7, ouabain-inhibitable K^+ uptake was markedly reduced. Comparison of total and ouabain-insensitive K^+ uptake in this study with that in Figure 6 revealed a marked drop in total and ouabain-sensitive and a substantial diminution in ouabain-insensitive K^+ uptake. Computer evaluation of these results yielded a total K^+ influx rate of 6.39×10^{-9} mEq $K^+/cm^2 \cdot min$, a ouabain-sensitive rate of 0.77×10^{-9} mEq $K^+/cm^2 \cdot min$, and a ouabain-insensitive rate of 5.62×10^{-9} mEq $K^+/cm^2 . min$.

In addition, it was determined that glucose concentration would not be expected to have significantly affected the results in the radiocation uptake experiments that follow since no reduction below 50 mg. per 100 ml. of medium was observed. For rat thymocytes, the average rate of glucose utilization was computed to be approximately 1×10^{-9} mg./cell/hour.

Effect of Ouabain on Intracellular Cation Concentration

A comparison of Figures 8 and 9 revealed a nearly reciprocal change in intracellular sodium and potassium ion concentrations in rat thymic and human CLL lymphocytes, respectively, when exposed to 5×10^{-3} M. ouabain *in vitro*. The curve in Figure 9

Figure 7. The effect of incubating rat thymocytes at 2° C. on K^+ influx. (The isolated cells were first pre-incubated at 37° C. for 60 minutes.

Abscissa: time (in minutes)

Ordinate: radiopotassium distribution ratio

Key: ○ total K^+ influx

● ouabain-insensitive K^+ influx

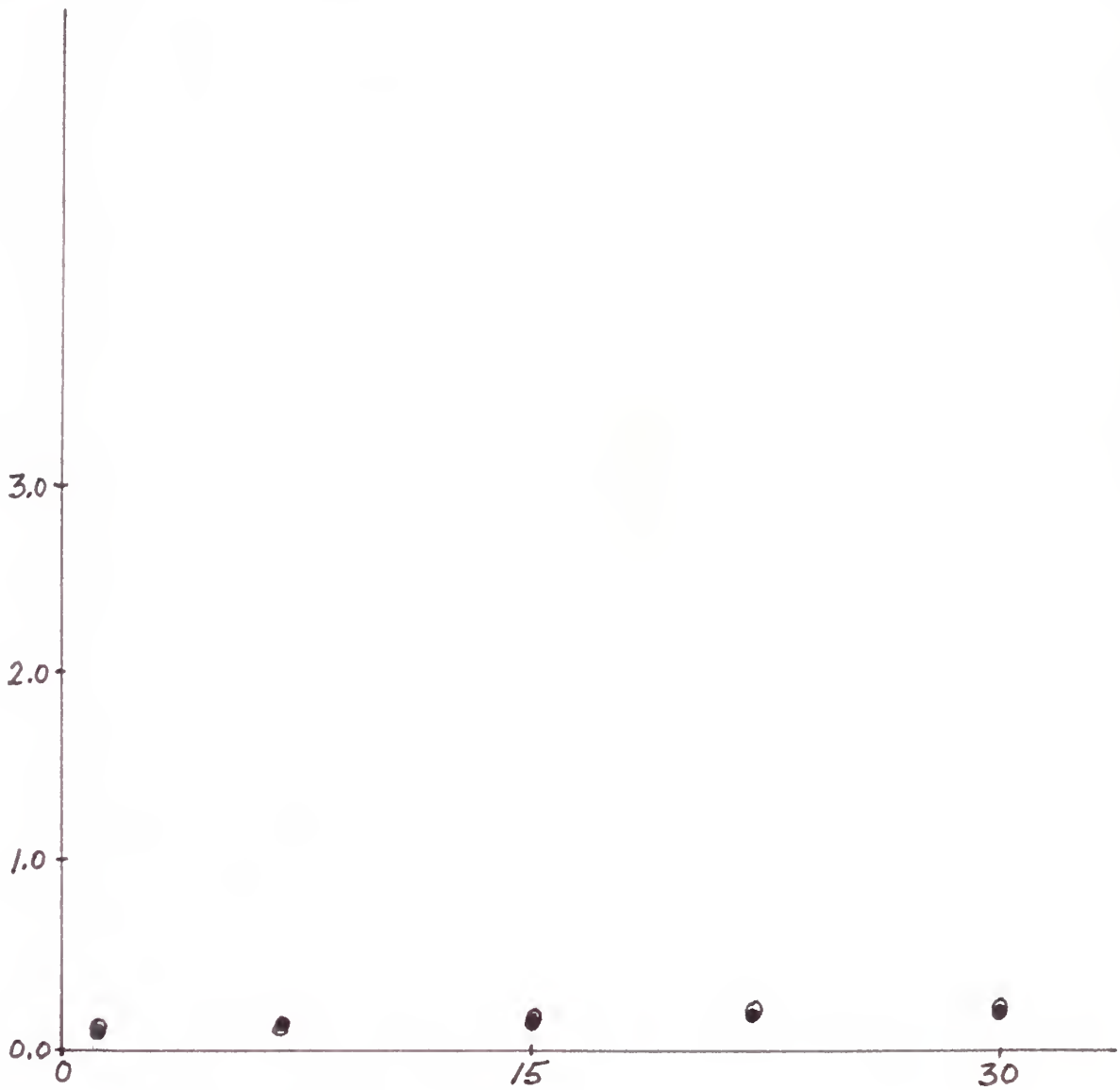

3.0
2.0
1.0
0.0
0
15
30

Figure 8. The effect of 5×10^{-3} M. ouabain on intracellular sodium and potassium ion concentrations in pooled rat thymocytes over four hours of incubation at 37° C.

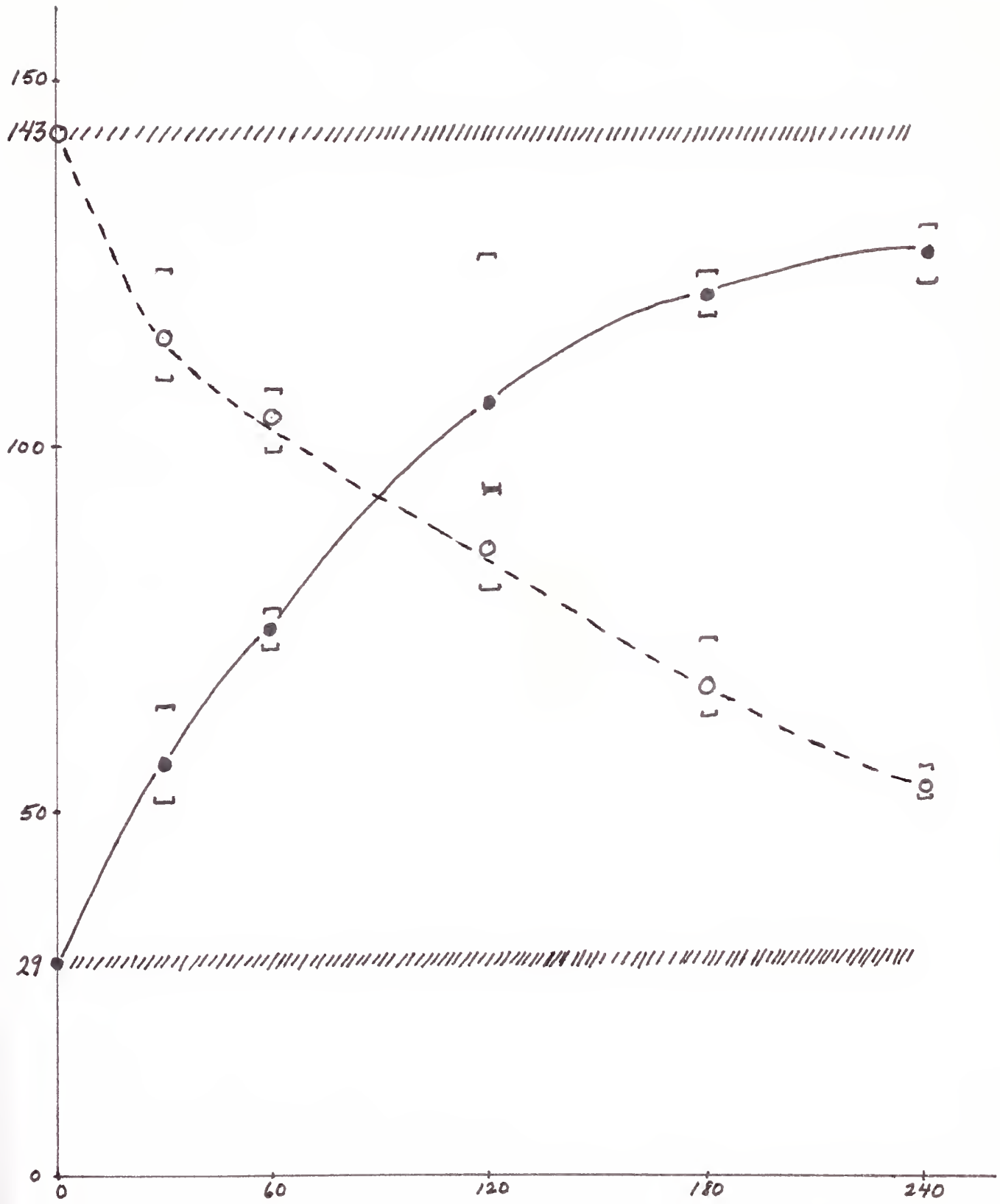
Abscissa: time (minutes)

Ordinate: intracellular ionic concentrations (in mEq/liter cell water)

Key: Na^+ (with range bars)

K^+ (with range bars)

(curve fitted by eye)


150
143
100
50
29
0
0
60
120
180
240

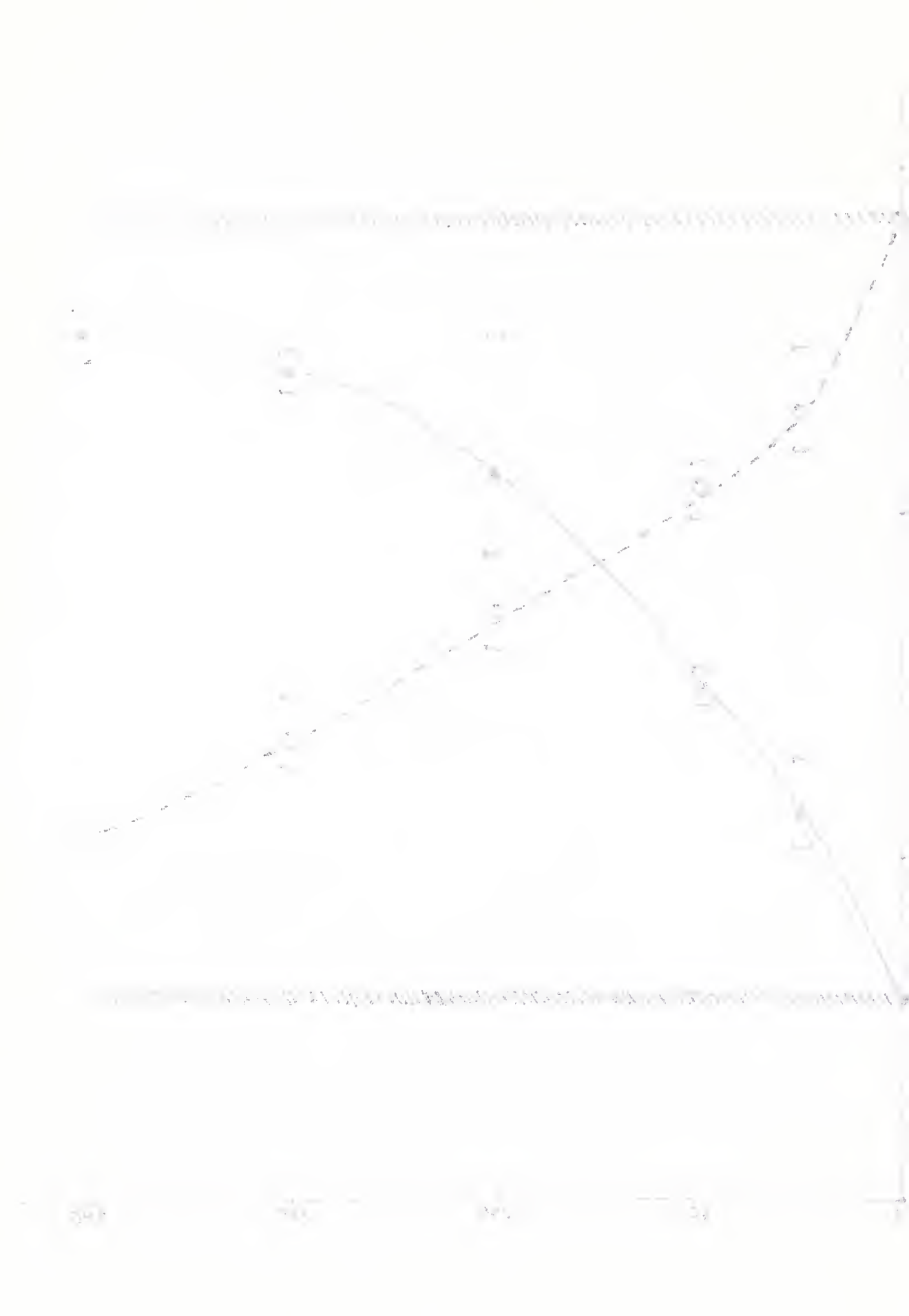


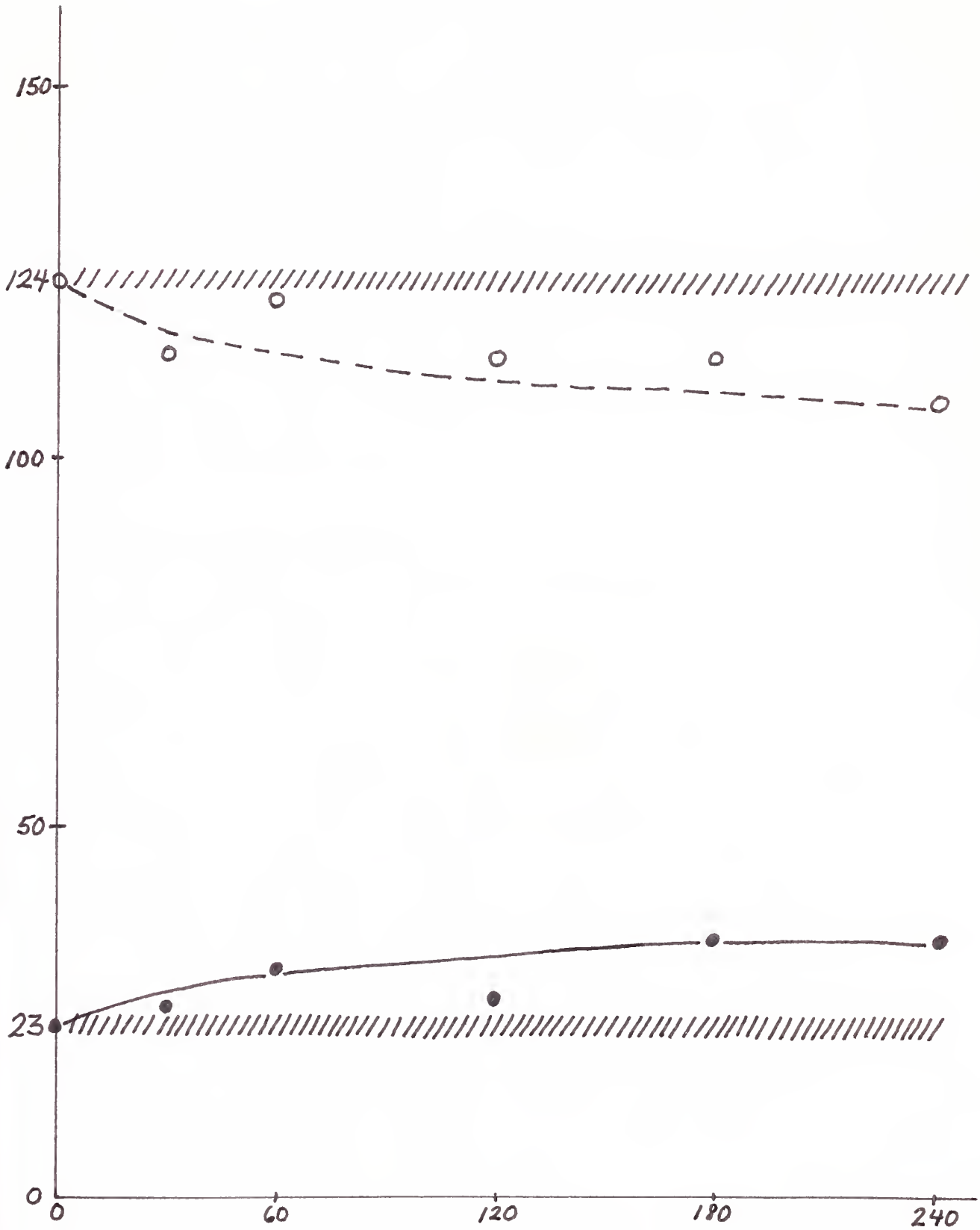
Figure 9. The effect of ouabain (5×10^{-3} M.) on intracellular sodium and potassium ion concentrations in human CLL lymphocytes over four hours of incubation at 37° C. (a representative curve drawn by eye)
Patient: V.S.

Abscissa: time (in minutes)

Ordinate: Intracellular cationic concentration (in mEq/liter cell water)

Key: ●—● Na^+

○--○ K^+


150
124
100
50
23
0
0
60
120
180
240

was representative of the effect of ouabain on one CLL patient's lymphocytes. Lack of a close fit of the latter curve in contrast to that in Figure 8 stemmed from there being only one observation per time period. These results also demonstrated that in the presence of ouabain intracellular monovalent cation concentrations underwent a much more rapid reduction toward intracellular levels in rat thymocytes than in human CLL lymphocytes. No study of this type was performed with human tonsillar lymphocytes.

In Vitro Radiopotassium Uptake

The radiopotassium distribution ratios plotted against time in the three types of lymphocytes were compared as in Figures 10 and 11. Figure 10 showed only minor differences in ouabain-uninhibitable uptake. In sharp contrast, Figure 11 revealed that ouabain-sensitive K^+ uptake was markedly lower in human CLL lymphocytes (B) than in rat thymic lymphocytes (A) and that uptake in human tonsillar lymphocytes (C) was even lower than that of CLL lymphocytes. Table 1.0 showing the corresponding computer-derived K^+-influx rates accompanied by the appropriate statistical analysis supported the above interpretation. Because of the inconsistency and wide range of measurements of intracellular water and cation content in human normal non-leukemic lymphocytes, total

Figure 10. Ouabain-insensitive K^+ influx in
A) rat thymocytes - mean ± S.E. for four experiments
B) human CLL lymphocytes - mean ± S.E. for four experiments (patients: F.A., G.B., H.R., V.S.)
C) human normal non-leukemic lymphocytes - mean with range of two experiments (patients: W.B., N.M.)

Abscissa: time (in minutes)

Ordinate: radiopotassium distribution ratio

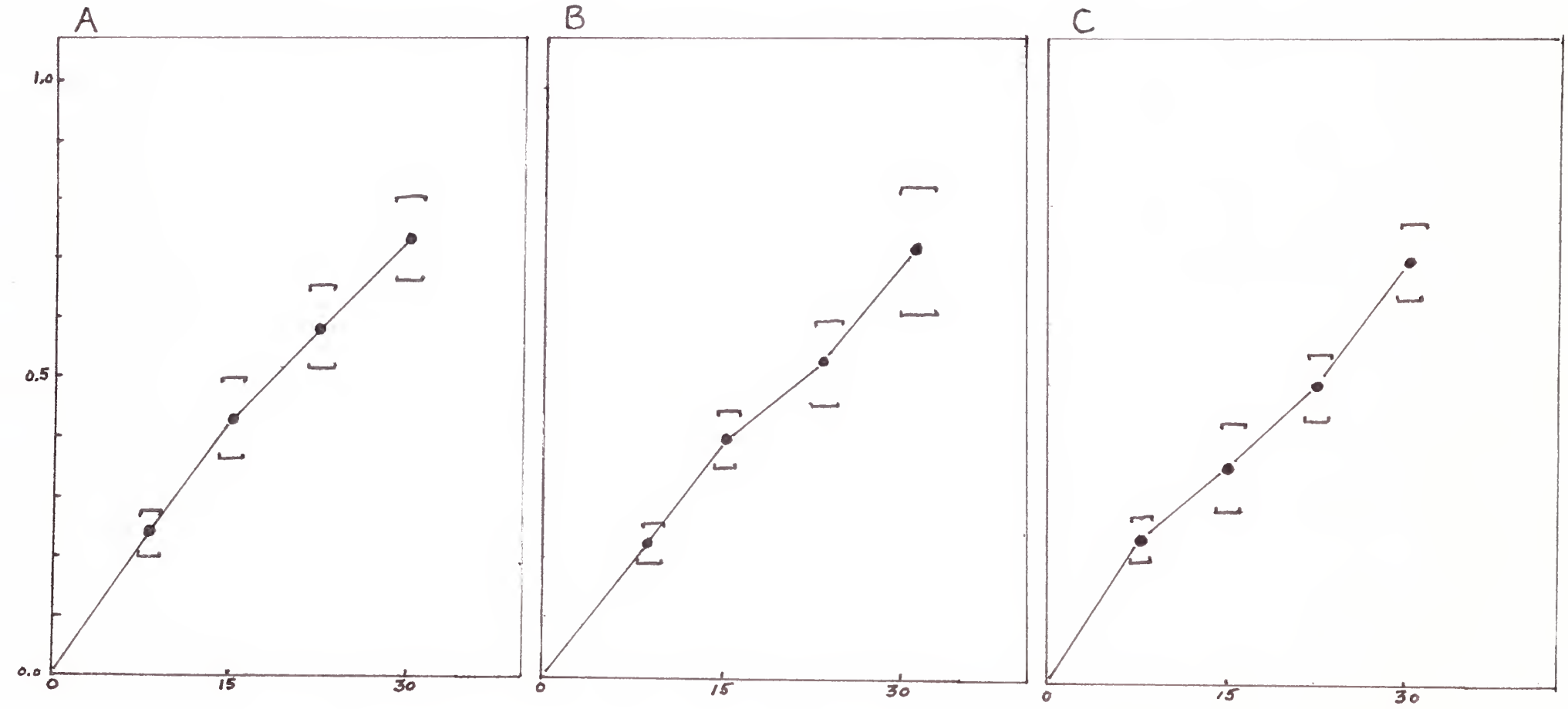
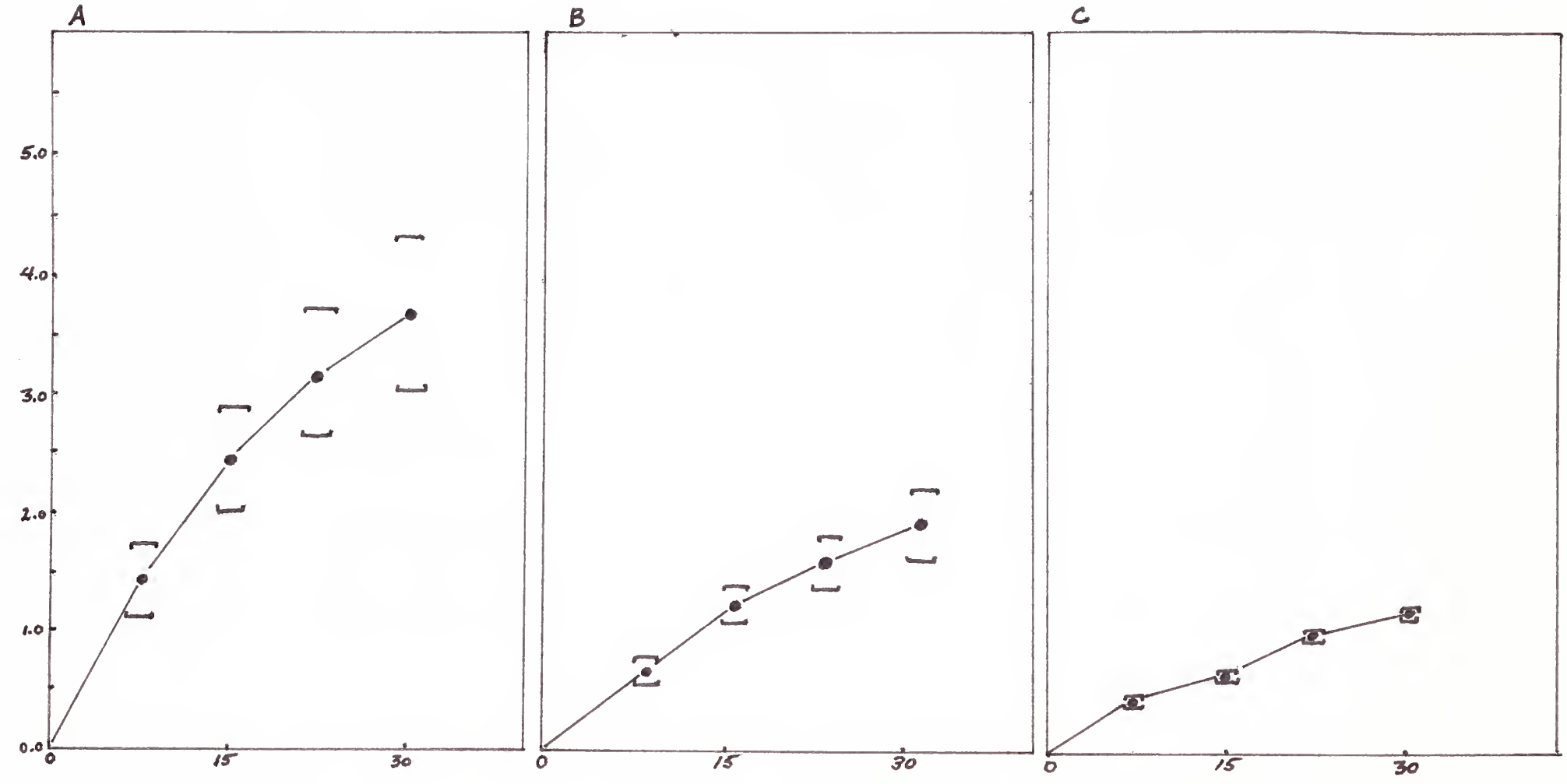

A
B
C
1.0
0.5
0.0
0
15
30
0
15
30
0
15
30

Figure 11. Ouabain-inhibitable K^+ influx in
A) rat thymocytes - mean ± S.E. for four experiments
B) human CLL lymphocytes - mean ± S.E. for four experiments (patients: F.A., G.B., H.R., V.S.)
C) human normal non-leukemic lymphocytes - mean with range of two experiments (patients: W.B., N.M.)

Abscissa: time (in minutes)

Ordinate: radiopotassium distribution ratio


A
B
C
5.0
4.0
3.0
2.0
1.0
0.0
0
15
30
0
15
30
0
15
30

Total K^+-Influx Rates*

	Rat Thymocytes (RT)		Human CLL Lymphocytes (CLL)
1.	6.71×10^{-8}	F.A.	3.89×10^{-8}
2.	6.17×10^{-8}	G.B.	4.39×10^{-8}
3.	10.42×10^{-8}	H.R.	3.70×10^{-8}
4.	17.09×10^{-8}	V.S.	5.39×10^{-8}
Mean :	10.10×10^{-8}		4.34×10^{-8}
± S.E.:	2.51×10^{-8}		0.38×10^{-8}

P = 0.11

Ouabain-Sensitive K^+-Influx Rates*

	RT		CLL
1.	5.29×10^{-8}	F.A.	2.54×10^{-8}
2.	5.42×10^{-8}	G.B.	3.51×10^{-8}
3.	9.04×10^{-8}	H.R.	2.50×10^{-8}
4.	15.03×10^{-8}	V.S.	4.41×10^{-8}
Mean :	8.70×10^{-8}		3.24×10^{-8}
± S.E.:	2.28×10^{-8}		0.91×10^{-8}

P = 0.10

Ouabain-Insensitive K^+-Influx Rates*

	RT		CLL
1.	1.42×10^{-8}	F.A.	1.35×10^{-8}
2.	0.75×10^{-8}	G.B.	0.88×10^{-8}
3.	1.38×10^{-8}	H.R.	1.20×10^{-8}
4.	2.06×10^{-8}	V.S.	0.98×10^{-8}
Mean :	1.40×10^{-8}		1.10×10^{-8}
± S.E.:	0.54×10^{-8}		0.21×10^{-8}

P = 0.38

TABLE 1.0. Computer-Derived K^+-Influx Rates

* in milliequivalents K^+/cm^2, min.

K^+-influx rates were determined by computer for intracellular potassium ion concentrations ranging from 98 to 139 mEq/liter cell water and for the proportion of cell water ranging from 0.78 to 0.81. This resulted in a range of computer-derived mean K^+-influx rates of from 2.35 to 2.37 x 10^{-8} mEq/cm^2·min, only a slight effect despite a wide range in corresponding K^+ concentrations. Thus, the total K^+-influx rate of human tonsillar lymphocytes was nearly half that of human CLL lymphocytes and one fourth that of rat thymocytes. As no experiment to determine the effect of ouabain on intracellular potassium ion concentration over a thirty-minute period was performed in human tonsillar lymphocytes, no computer assessment could be made of the ouabain-sensitive and -insensitive K^+-influx rates. By calculating the mean thirty-minute ouabain-unihibitable $^{42}K^+$ distribution ratio and dividing by the corresponding value for total K^+ uptake, the proportion of the total K^+ uptake that was ouabain-insensitive, or passive, influx was obtained. Thus passive K^+ influx represented approximately 16.7% of total K^+ influx in rat thymocytes, 27.5% in human CLL lymphocytes, and 38% in human normal non-leukemic lymphocytes. These values correlated well with the computer-derived ones of 13.9% for rat thymocytes and 25.4% for human CLL lymphocytes; these values were slightly lower as expected since

the computer program took $^{42}K^{+}$ efflux into consideration.

In Vitro Radiosodium Uptake

Sodium-22-uptake studies demonstrated that 5×10^{-3} M. ouabain significantly increased the radiosodium distribution ratio as compared with the control cell suspension in all three groups of lymphocytes (see Figure 12). The degree of increase paralleled that found in the $^{42}K^{+}$ uptake studies above although the interpretation of the plot in Figure 12C depicting uptake in tonsillar lymphocytes was not altogether clear. The $^{22}Na^{+}$ distribution ratio did not appear to be leveling off at about the 0.1 point in the untreated group as it did in the case of both rat thymocytes and human CLL lymphocytes. However, a significant difference in radiosodium distribution ratios was noted for human normal non-leukemic lymphocytes.

In Vivo Radiocation Uptake in Rat Thymus

The above in vitro $^{42}K^{+}$- and $^{22}Na^{+}$-uptake studies would not have been complete without knowing what the distribution ratio of these monovalent cations was in vivo. Such studies as described previously under METHODS were performed. Thirty minutes following intraperitoneal injection of $^{42}K^{+}$, the mean $^{42}K^{+}$ distribution between rat thymus cells and serum was 11.96 and, after three hours,

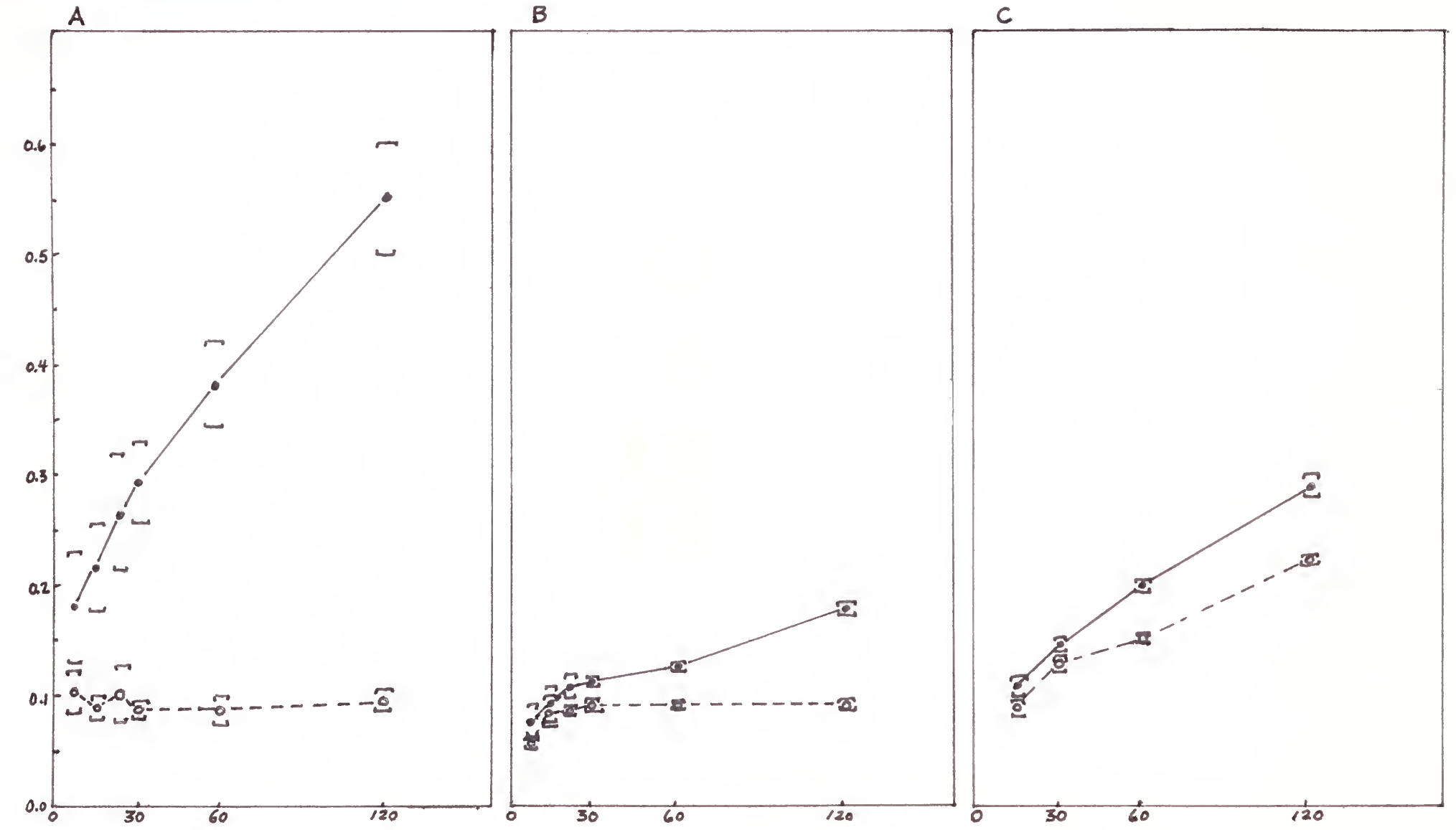
Figure 12. Total and ouabain-insensitive Na^+ influx in
A) rat thymocytes - mean ± S.E. of three experiments
B) human CLL lymphocytes - mean ± S.E. of three experiments (patients: V.S., J.B., Mi.M.)
C) human normal non-leukemic lymphocytes - mean ± S.E. of three experiments (patients: Ma.M., T.D., L.W.)

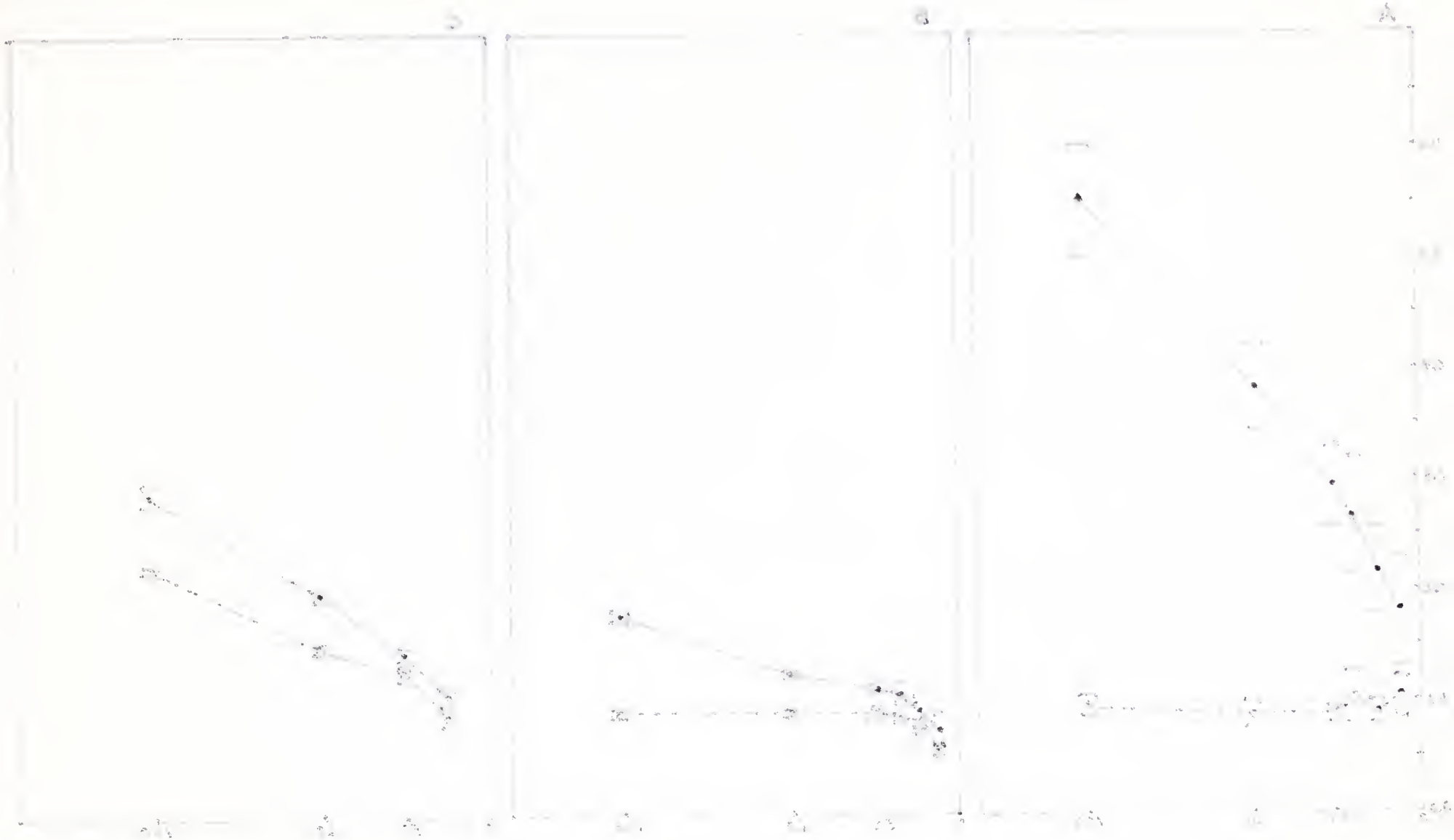
Abscissa: time (in minutes)

Ordinate: radiosodium distribution ratio

Key: O ---- O total (control)

●——● ouabain-insensitive


A
B
C
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
120



23.64. Using a mean intracellular potassium ion concentration in rat thymus of 143 mEq/liter cell water based on measurements in thymus, the mean thymus : serum distribution ratio of non-radioactive potassium in four rats was 22.7. Thus, by 30 minutes *in vivo* the radiopotassium distribution ratio reached approximately 50% of the non-radioactive K^+ distribution ratio and 100% at some time before three hours. Comparing this *in vivo* data with those *in vitro*, it was found that the level reached in rat thymocytes *in vitro* in 30 minutes (4.38 ± S.E. of 0.713) was approximately 50% of that *in vivo* after the same period of time.

Similar studies were performed using $^{24}Na^+$. After three hours, the radiosodium ratio reached 0.196. Using an intracellular sodium ion concentration in rat thymus of 29 mEq/liter cell water as measured experimentally, the non-radioactive sodium distribution between rat thymus tissue and serum was 0.181 (mean of three rats). This indicated that the control Na^+ distribution ratio was reached, if not slightly exceeded, by three hours. In contrast, the *in vitro* $^{22}Na^+$ distribution ratio was found to be 0.103 (mean of four experiments) ± a standard error (S.E.) of 0.0163--approximately 57% of the expected ratio. Indeed this ratio had been recorded within the first 10 minutes of the *in vitro* study.

Effect of Oligomycin on In Vitro Radiopotassium Uptake

Oligomycin had no effect on $^{42}K^{+}$ uptake in rat thymocytes at concentrations of 5 and 50 µg/ml. As observed from Figure 13, treatment with 500 µg oligomycin/ml. (1.25×10^{-5} M) produced a significant, though small, absolute reduction in the total $^{42}K^{+}$ distribution ratios in rat thymocytes and in human normal non-leukeumic lymphocytes whereas human CLL lymphocytes appeared much more sensitive to oligomycin. That percentage of the total $^{42}K^{+}$ influx that was oligomycin-sensitive was 12.2% for rat thymocytes, 64.5% for CLL lymphocytes, and 10.8% for tonsillar lymphocytes. That percentage of the ouabain-sensitive (active) $^{42}K^{+}$ influx that was oligomycin-sensitive was 14.3% for rat thymocytes, 77.5% for CLL lymphocytes, and 26.6% for NNL lymphocytes.

Effect of Cortisol and Cycloheximide on In Vitro Radiopotassium Uptake in Rat Thymocytes

After three hours of incubation with 10^{-5} M. cortisol, there was no statistically significant effect on either total, ouabain-inhibitable, or ouabain-uninhibitable K^{+} uptake as compared with the control (see the two lefthand columns of Figure 14). The means and standard deviations of the radiopotassium distribution ratios of the respective categories of the control

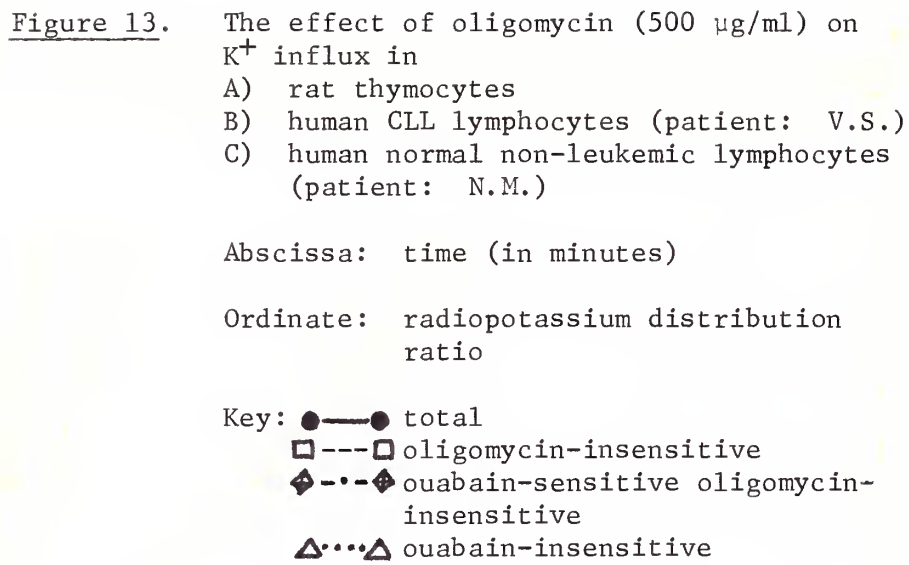


Figure 13.
The effect of oligomycin (500 µg/ml) on K^+ influx in
A) rat thymocytes
B) human CLL lymphocytes (patient: V.S.)
C) human normal non-leukemic lymphocytes (patient: N.M.)
Abscissa: time (in minutes)
Ordinate: radiopotassium distribution ratio
Key:
total
oligomycin-insensitive
ouabain-sensitive oligomycin-insensitive
ouabain-insensitive

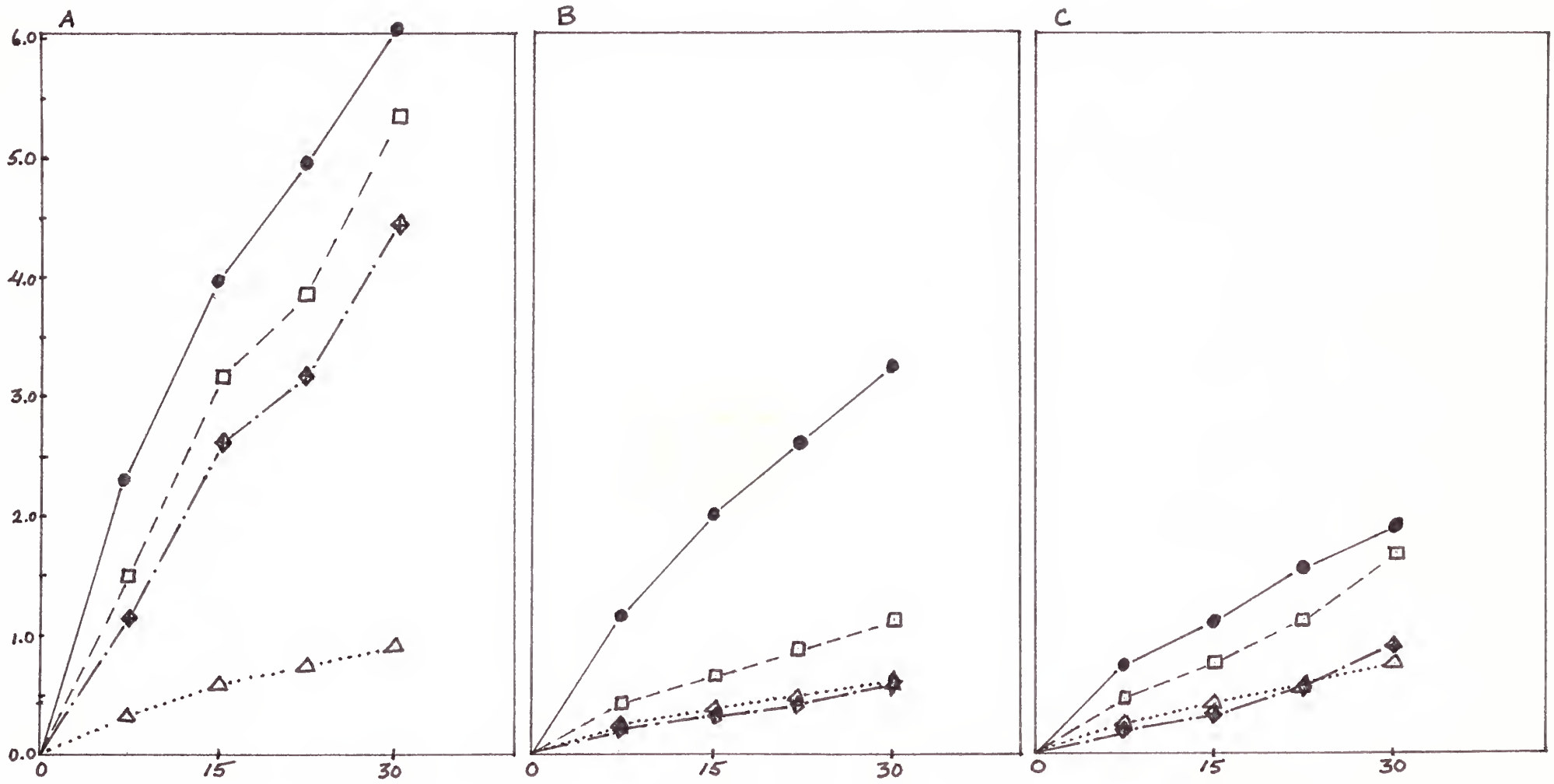

A
B
C
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
15
30
0
15
30
0
15
30

Figure 14. The effect of cortisol and cycloheximide on K^+ uptake in rat thymocytes

Abscissa: as shown

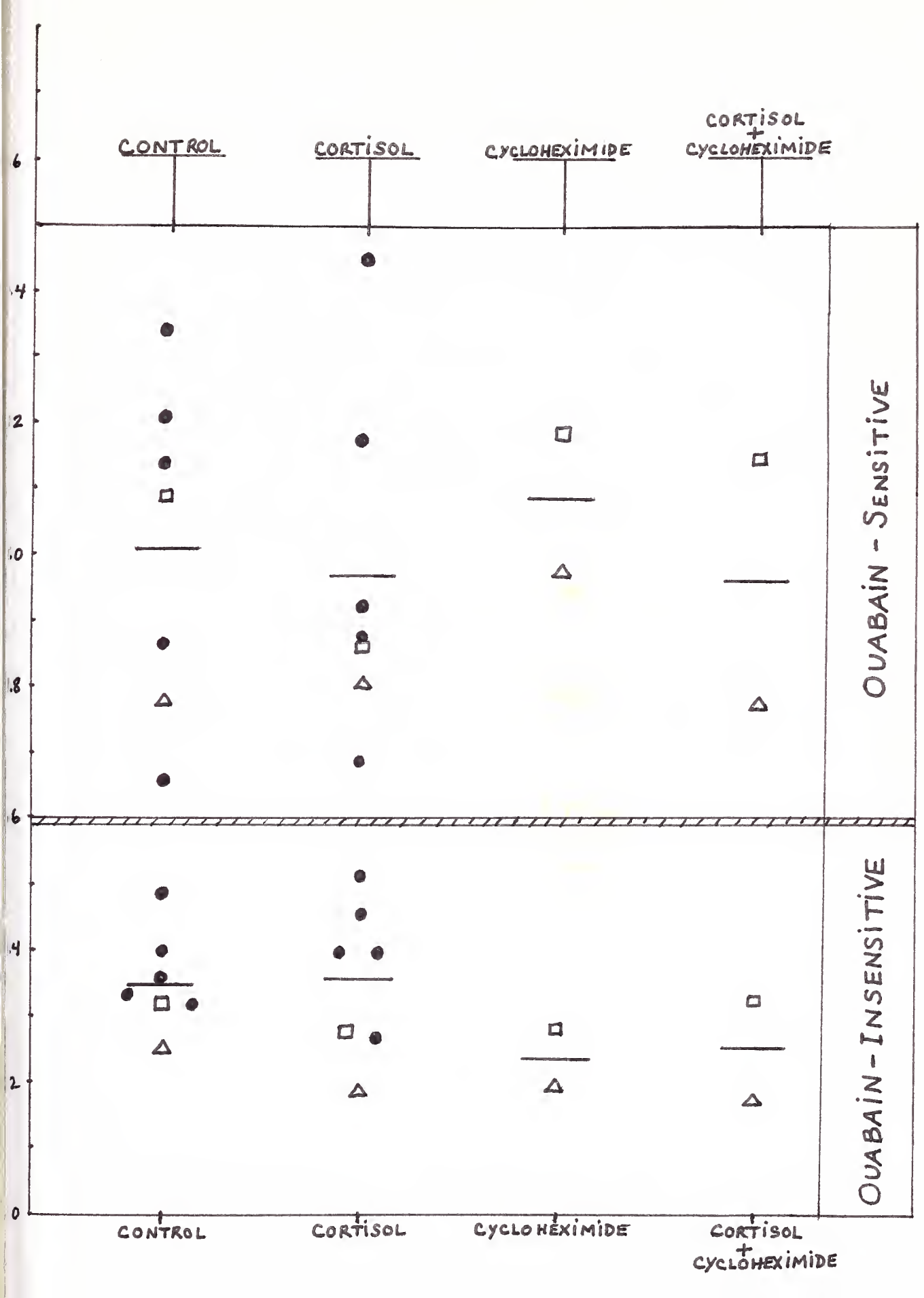
Ordinate: radiopotassium distribution ratio

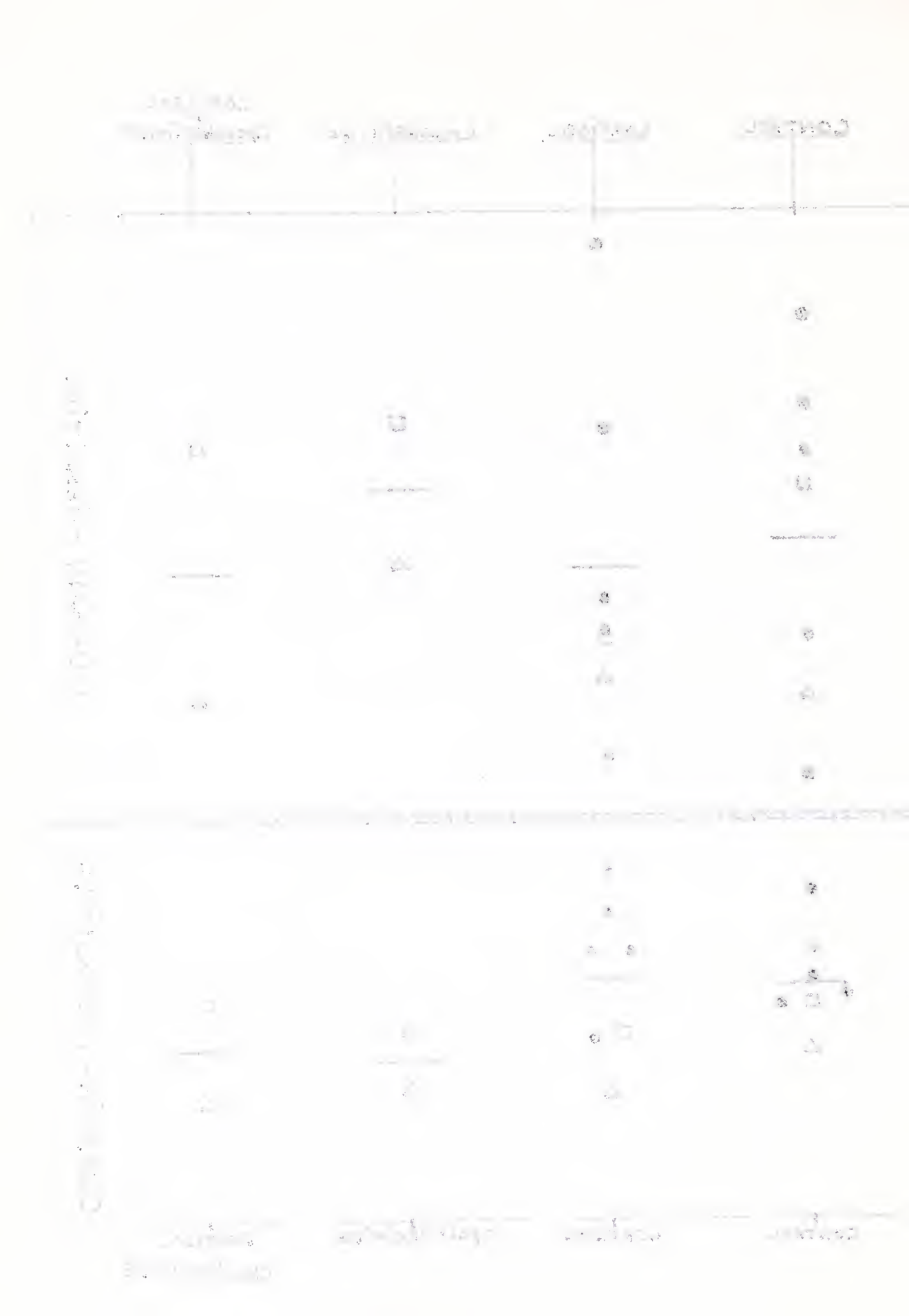
Key: ● represents an experiment in which there was only an untreated (control) and a cortisol-treated sample

□,△ represent two separate experiments in which untreated (control), & cortisol-, cycloheximide-, and cortisol +cycloheximide-treated samples were run simultaneously

●,□,△ represent mean of triplicate aliquots

——— represents the mean of 7 or 2 experiments as applies


CONTROL
CORTISOL
CYCLOHEXIMIDE
CORTISOL + CYCLOHEXIMIDE
6
4
2
1.0
.8
.6
.4
.2
0
OUABAIN - SENSITIVE
OUABAIN - INSENSITIVE
CONTROL
CORTISOL
CYCLOHEXIMIDE
CORTISOL + CYCLOHEXIMIDE



versus the cortisol-treated group were as follows: total, 1.37 ± 0.31 vs. 1.32 ± 0.34; ouabain-sensitive, 1.01 ± 0.25 vs 0.97 ± 0.26; and ouabain-insensitive, 0.35 ± 0.08 vs. 0.36 ± 0.12. Statistical analysis of the difference between the means using the Student t Test yielded P values of 0.80, 0.77, and 0.90, respectively. In addition, as expected, it was found that the 10-minute radiopotassium distribution ratios were entirely consistent with those cited earlier in the 30-minute $^{42}K^{+}$-uptake studies.

Two experiments were done(as shown by the open squares and triangles of Figure 14) to determine if cortisol alone, cycloheximide alone, or both together would have any effect on $^{42}K^{+}$ uptake or if cycloheximide would cancel the effect of cortisol. No significant effect was noted.

DISCUSSION

Little work has been done to examine the characteristics of monovalent cation movements across lymphocyte membranes. Great difficulty had arisen with the isolation of a sufficient quantity of cells for analysis and of cells that were not influenced by chemical and physical trauma in the process. The latter impediment to research may well have accounted for the lower intracellular potassium ion concentration and higher intracellular sodium ion concentration recorded for human CLL lymphocytes by Rigas (22) as compared with the foregoing results presented here. The value given above for the mean cell volume of human tonsillar lymphocytes of 294 μ^3 correlated well with the estimated mean cell volume of normal circulating small lymphocytes of 269 μ^3 given by Lichtman and Weed (21) especially when the evidence for greater morphologic homogeneity of circulating small lymphocytes versus the substantial proportion of blastic-appearing lymphocytes isolated from tonsillar tissue (cf. Piffkó et al. (39)) is taken into consideration.

Conversion of the total K^+-influx rate for human erythrocytes given by Solomon and Gold (40) to the same units as in the data presented here demonstrated that the total K^+-influx rate in human

lymphocytes is approximately one order of magnitude greater than that in the human red cell (20 - 40 x 10^{-9} mEq $K^+/cm^2 \cdot min$ vs. 3.86 x 10^{-9} mEq $K^+ cm^2 \cdot min$*). It must be acknowledged, of course that the lymphocyte cell-surface-area estimate was based on the assumption of a smooth sphere because the area of its villous surface cannot be accurately calculated (41, 42). Indeed, if these surface projections were taken into account in the flux computations, values perhaps one third of those shown here might be forthcoming. However, this would still indicate a three-fold greater flux rate than that in erythrocytes. This contingency was dealt with by Lamb and MacKinnon (43) in their studies of sodium and potassium movements in L cells. In any case, a nearly proportionate increase in cell surface area in the three types of lymphocytes would be expected if the microvilli were considered.

Block and Bonting (20) and Lichtman and Weed (21) found that the (Na^+-K^+)-activated membrane ATPase activity of human normal and CLL lymphocytes were similar. A comparison of these

*Assume $V_{c_{RBC}} = 86\mu^3$, $A_{c_{RBC}} = 140\mu^2$, % cell H_2O = 68.

findings with the results of the $^{42}K^{+}$-uptake studies discussed here indicate that there is no direct correlation between the $(Na^{+}-K^{+})$-activated ATPase activity of the cell membrane fragments with the actual quantitative movement of monovalent cations across the intact cell membrane. Rather, it may well indicate only that these two types of human lymphocytes, if not all mammalian lymphocytes, have the same number of ouabain-inhibitable sites per unit surface area of membrane; how primed these sites are may depend upon the degree of membrane permeability, i.e., upon the magnitude of non-energy dependent, non-enzymatically catalyzed, passive fluxes of sodium and potassium.

Why was there an apparent discrepancy between the in vivo and in vitro radiocation uptake studies in rat thymocytes? For the same period after radiocation injection, the total cation distribution ratios for both Na^{+} and K^{+} in the in vitro studies were roughly half those of the in vivo, the theoretical ratios. Of course, it could not be known whether the ouabain-uninhibitable influx rates were the same. A difference in active transport rates, or ouabain-sensitive influx rates, might be expected if one postulated that the cell were operating at a generally lower metabolic rate in vitro. The latter might be explainable on the basis of physical and/or chemical damage suffered by the

rat thymocytes during isolation or the lack of an adequate pO_2 level *in vitro*. This would explain the $^{42}K^+$-uptake data difference but not the radiosodium-uptake data difference. The reasons are that sodium-ion influx is a passive, non-energy-dependent process (3, 4, 8, 9) and damage to the pump would have resulted in a higher intracellular Na^+ concentration (an altered steady-state level), thus leading one to expect to find a higher total radiosodium distribution ratio. Therefore, damage to the structural elements of the plasma membrane must be postulated to have occurred as well. The finding that the results of the *in vivo* and *in vitro* $^{42}K^+$-uptake studies were of the same relative magnitude was encouraging, however. In any case, this points up the fact that it is difficult to make an exact comparison between *in vivo* and *in vitro* experiments on the same cells since all the physiologic parameters cannot as yet be controlled.

The experiments presented above lend strong support for the existence of an active linked Na-K pump in the lymphocyte plasma membrane. All three groups of lymphocytes were sensitive to 5×10^{-3}M. ouabain and to 1.25×10^{-5}M. oligomycin. Interestingly enough, the concentrations of both inhibitors appear roughly fifty times that required to exert a near maximal effect in human erythrocytes (3, 4, 10, 11, 44). The time-course studies demonstrated a

reciprocal loss of potassium and gain of sodium upon incubation with 5×10^{-3}M. ouabain in both rat thymocytes and human CLL lymphocytes. This was further confirmed by the radiopotassium- and radiosodium-uptake studies in which the magnitude of absolute reduction in the thirty-minute $^{42}K^{+}$ distribution ratios reflected that of the increase in the thirty-minute $^{22}Na^{+}$ distribution ratios with ouabain treatment. In other words, the percentage of total cation uptake that consisted of a ouabain-inhibitable (active) component was greatest in rat thymocytes, lower in human CLL lymphocytes, and least in human normal non-leukemic (tonsillar) lymphocytes. These results are consistent with an energy-dependent, ouabain- and oligomycin-sensitive (Na^{+}-K^{+})-activated membrane ATPase in lymphocytes similar to that in red cells. Studies of the effect of such inhibitors of oxidative phosphorylation as 2,4-dinitrophenol and KCN and inhibitors of anaerobic glycolysis, e.g., sodium iodoacetate and NaF (low concentrations) (45), on membrane transport of Na^{+} and K^{+} in lymphocytes as has been performed in red cells are needed to define the energy-dependent nature of the Na-K pump in the lymphocyte.

The above data are in direct support of the work done by Wilson and Manery (13) and Hempling (14) and in contradistinction

to that of Elsbach and Schwartz (15) (see INTRODUCTION). Since Woodin and Wieneke's (17, 18) argument (see INTRODUCTION) for a membrane K^+ pump not linked to Na^+ transport in leukocytes relates to neutrophils, not lymphocytes, no refutation can be made of their evidence from these studies. However, preliminary studies not presented here indicated that in human acute leukemic myeloblasts both ouabain- and oligomycin-inhibitable $^{42}K^+$ uptake were observed in vitro. More work must be done before a firm statement can be made concerning granulocytes.

In the work of Quastel and Kaplan (23-25), reviewed earlier, it seems unlikely that ouabain, in the low concentrations used in their experiments, exerted its effect via an inhibition of (Na^+-K^+)-activated membrane ATPase. No effect was seen in human CLL lymphocytes with ouabain concentrations below 10^{-5}M. It appears probable, then, that long-term incubation with low concentrations of ouabain may have some other as yet unexplainable deleterious effect on lymphocytes. A direct nuclear effect by the steroid moiety of ouabain as suggested by the authors is one possibility.

While on the subject of steroids, consider the cortisol experiments presented earlier. These experiments clearly demonstrated that 10^{-5}M. hydrocortisone treatment of rat thymocytes

for three hours in vitro produced no statistically significant diminution in $^{42}K^{+}$ uptake. This contradicts the observations by Makman and associates (42) showing a 20% decrease in rubidium-86 uptake after three hours of cortisol treatment in vitro.

There appears to be a species difference in lymphocyte permeability characteristics. There may also be a neoplastic difference. No significant difference was found among the rates of ouabain-insensitive (passive) K^{+} influx in the three lymphocyte types. However, significant differences were discovered among the ouabain-sensitive (active) K^{+}-influx rates. In order to maintain steady-state conditions with respect to intracellular K^{+} concentration, the rate of K^{+} efflux, a passive process based upon the permeability properties of the membrane (9), must follow the same order: rat thymocytes>human CLL lymphocytes>human tonsillar lymphocytes. Although radiopotassium-efflux studies were not performed to confirm this, the non-radioactive time-course studies with ouabain (Figs. 4, 5) indicated that K^{+} does leave the intracellular milieu more rapidly in rat thymocytes than in CLL lymphocytes. Hence, passive K^{+} efflux is more rapid in the rat thymocyte than in the CLL lymphocyte; the rat thymocyte membrane is more permeable to K^{+} as seen from its inner aspect in contradistinction to the CLL lymphocyte membrane. These non-

radioactive studies also supported the inference from the radio-sodium-uptake studies that the rate of passive Na^+ influx is greater in rat thymocytes than in CLL lymphocytes. Whether active (ouabain-sensitive) Na^+ efflux was likewise greater could only be decuced from, not proven by, these studies. Studies of active and passive $^{22}Na^+$-efflux rates in the three cell types would be worthwhile.

These studies suggest that an important difference between normal and leukemic (neoplastic) human lymphocytes may lie in their membrane permeabilities to intracellular K^+ and extracellular Na^+. If membrane permeability to Na^+ and K^+ were increased, one could easily imagine that the Na-K pump would then be operating at a more highly primed level in order to maintain steady-state conditions. Is the initial step in the transformation of a normal clone of cells to a neoplastic state of proliferation a subtle alteration of membrane permeability characteristics? Many studies on various cell lines have been performed that are consistent with this theory. Lubin and his colleagues showed that lowering the intracellular potassium concentration in a mutant strain of Escherichia coli B (46) and in amphotericin-treated Sarcoma-180 cells (47) produced a significant decrease in endogenous protein and nucleic acid synthesis. Lubin suggested

that intracellular potassium concentration acted as a metabolic pacemaker in bacterial and animal cells. Working with mouse leukemic lymphoblasts in tissue culture, Jung and Rothstein (48) found that the growth and division cycle of these cells was accompanied by changes in intracellular sodium and potassium ion concentrations that followed an as yet unexplainable pattern. Kalant and Hickie (49) found that net K^+ uptake and the initial intracellular K^+ concentration were higher in Morris hepatoma 5123tc tissue than in normal rat liver. Yunis and his colleagues (50) have demonstrated that sodium was required for active transport of amino acids in human leukocytes, that maximal rates of transport required optimal intracellular sodium and potassium ion concentrations, and that ouabain inhibited this transport to a variable extent. Mayhew and Levinson (51) recently discovered that 6×10^{-4}M. ouabain inhibited both cation transport, as expected, and cell division in Ehrlich mouse ascites tumor cells. Christensen and Henderson (52) have suggested that neoplastic cells differed from normal cells in that they had a greater potential for the accumulation of amino acids; this increase in amino-acid pool size permitted rapid growth of the cell. Marvin (53) suggested that the membrane might be the key to the control of DNA replication in his presentation of the "site-territory" model as a basis for this control. The implications raised above

are consistent with Wallach's (54) hypothesis that the oncogenic agent acts to insert an inappropriate protein into the plasma membrane of cells of a normal clone, thus producing a permanent change in the antigenicity, permeability, and biophysical and bioelectric surface characteristics of the membrane. An alteration of membrane structural protein might then have a subtle, though crucial, effect on the metabolic rate of such transformed cells. Hence, an alteration of rates of transmembranous movement of sodium and/or potassium ions may well be associated with the genesis of a neoplasm. As suggested a decade ago in a review by Abercrombie and Ambrose (55), further comparative studies of tumor-cell lines and their normal counterparts should prove to be extremely enlightening and expand our understanding of the intricacies of the neoplastic process, especially when still more sophisticated techniques for doing so become available.

SUMMARY

The monovalent cation metabolism of rat thymocytes and human normal and leukemic lymphocytes has been studied in a preliminary fashion.

Differences in mean cell volumes of lymphocytes of three diverse cell lines were described. Rat thymocytes, human CLL lymphocytes, and human tonsillar lymphocytes all possessed cation membrane transport systems sensitive in varying degrees to ouabain and oligomycin. 5×10^{-3}M. ouabain produced a reciprocal decrease in intracellular K^+ concentration and increase in intracellular Na^+ concentration in both rat thymocytes and human CLL lymphocytes. It would appear that the lymphocyte membrane possesses an active Na-K pump.

Passive K^+-influx rates were similar in all three cells. Active K^+-influx rates were highest in rat thymocytes, intermediate in CLL lymphocytes, and lowest in human non-leukemic lymphocytes. Non-radioactive time-course studies with ouabain revealed that passive K^+-efflux and passive Na^+-influx rates were higher in rat thymocytes than in CLL lymphocytes. Results of radiosodium-uptake studies implied that active Na^+-efflux rates followed the same pattern as for active K^+-influx rates. The conclusion was drawn that rat thymocytes and human CLL and tonsillar lymphocytes differed

with respect to interior membrane permeability to K^+ and external membrane permeability to Na^+. Thus, with respect to monovalent cations, lymphocytes of rats and humans and of normal and neoplastic clones have dissimilar membrane permeability characteristics.

Prior treatment of rat thymocytes for three hours in vitro with 10^{-5}M. hydrocortisone alone, cycloheximide alone, and with both produced no significant alteration of K^+ uptake.

Implications of these studies were discussed.

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